

METHODS OF ADMINISTERING DALBAVANCIN FOR TREATMENT OF BACTERIAL INFECTIONS

FIELD OF THE INVENTION

[0001] This application relates to dalbavancin compositions and methods of use of such compositions in methods of treatment of bacterial infections.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application is a continuation of U.S. Application Serial No. 10/714,261, filed November 14, 2003, which claims the benefit of U.S. Provisional Patent Application Serial Nos. 60/427,654, filed November 18, 2002, 60/485,694, filed July 8, 2003, 60/495,048, filed August 13, 2003, and 60/496,483, filed August 19, 2003, the disclosures of all of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

[0003] According to the U.S. Center for Disease Control and Prevention, nosocomial bloodstream infections are a leading cause of death in the United States. Approximately five percent of the seven million central venous catheters (CVCs) inserted annually in the United States are associated with at least one episode of bloodstream infection (approximately 350,000 a year). Catheter-related bloodstream infections occur when bacteria enter the bloodstream through an intravenous catheter and can be life threatening.

[0004] Skin and soft tissue infections (SSTIs) are a common medical condition and often the consequence of trauma or surgical procedures. *Staphylococcus aureus* and *Streptococcus pyogenes* are the pathogens most frequently isolated from patients with deep tissue infections, although any pathogenic organism, including those found on healthy skin, may cause infection. Many SSTIs are mild to moderate in severity, permitting successful treatment with oral antimicrobial agents and local cleansing. In

contrast, more severe or complicated infections, which frequently occur in patients with underlying risk factors (*e.g.*, vascular compromise, diabetes) and/or infections caused by difficult-to-treat or multiply-resistant bacteria, may require potent intravenous antimicrobial therapy and aggressive surgical debridement.

[0005] Staphylococci are a clinical and therapeutic problem and have been increasingly associated with nosocomial infections since the early 1960s. The coagulase-positive species methicillin-resistant *Staphylococcus aureus* (MRSA) has long been problematic in both community-acquired and nosocomial infections, and several coagulase-negative staphylococci have been recognized as opportunistic human pathogens, especially in the treatment of critically ill patients in intensive care units. Another major cause for clinical concern is the increasing isolation of penicillin-resistant *Streptococcus pneumoniae* strains in many parts of the world.

[0006] The glycopeptide antibiotics vancomycin and teicoplanin have been used against serious nosocomial infections caused by multi-drug-resistant Gram-positive pathogens, particularly MRSA, coagulase-negative staphylococci (CoNS), and enterococci. Vancomycin and teicoplanin are used for infections caused by MRSA, and until recently, all isolates were uniformly susceptible. However, the isolation of *Staphylococcus aureus* strains with intermediate susceptibility or resistance to teicoplanin as well as vancomycin has now been reported with increasing frequency. A number of vancomycin-resistant strains, classified "VanA," "VanB," or "VanC," based on the mechanism of resistance, have been reported. Thus, alternative treatment options are needed.

[0007] Teicoplanin is at least as active as vancomycin against most Gram-positive bacteria and appears to cause fewer adverse events. Both forms of treatment require at least once daily dosing to effect complete recovery. Currently, the therapeutic options for severe infections caused by some of these pathogens is quite limited. The emerging resistance of Gram-positive pathogens to vancomycin makes the availability of new antibiotics with potential for increased effectiveness highly desirable.

[0008] In addition, less frequent dosing regimens than currently-available therapies would be desirable to enhance patient comfort, especially for parenteral, *e.g.*, intravenous or intramuscular, antibiotic administration. Hospital stays are sometimes necessitated by the need for multi-daily antibiotic administration by parenteral means, and less frequent dosing would be advantageous to permit such treatment to be done on an outpatient basis.

[0009] Although less frequent dosing is a desirable feature of an antibiotic administration regimen, the “pharmaceutical window,” *i.e.*, the toxicity profile, of the administered antibiotic must be sufficiently acceptable to permit a large single dose to be administered without jeopardizing treatment by causing severe adverse reactions in the treated patient. Further, even when an antibiotic exhibits a suitable pharmaceutical window, less frequent dosing is possible only if the antibiotic exhibits a suitable serum half-life to maintain therapeutic effectiveness over the dosing interval desired. The serum half-life of an antibiotic dictates both the longevity of a drug *in vivo* and the length of time after administration when the serum level will reach a minimum trough level which is still bactericidally effective. The serum trough level over time after administration of a first dose of antibiotic dictates when a further dose must be administered to retain a minimum bactericidal level of the antibiotic *in vivo*.

[0010] In view of the above, an antibiotic possessing activity against one or more antibiotic resistant bacterial strains, particularly MRSA, which could be administered at a dosing interval of once every 5-7 days or longer, would be of commercial value and would satisfy a long-felt need in the art.

SUMMARY OF THE INVENTION

[0011] The invention provides compositions, methods and kits for treatment or prevention of a bacterial infection with dalbavancin. Surprisingly, stabilized formulations of dalbavancin have been found to exhibit both a pharmaceutical

window as well as a prolonged serum half-life to permit treatment regimens of about once every 5-7 days or longer, while retaining antibacterial properties *in vivo*.

[0012] Accordingly, in one aspect, a pharmaceutical composition is provided that includes a unit dose of dalbavancin in an amount sufficient to provide a therapeutically or prophylactically effective plasma level of dalbavancin in an individual for at least five days, a stabilizer, and a pharmaceutically acceptable carrier.

[0013] Pharmaceutical compositions of the invention are generally formulated in a pharmaceutically acceptable form for administration to an individual, such as a pharmaceutically acceptable aqueous formulation. Such pharmaceutical compositions are preferably administered by parenteral, *e.g.*, intravenous or intramuscular, routes. Accordingly, in this preferred embodiment, these pharmaceutical compositions are typically sterile.

[0014] In some embodiments, a unit dose of dalbavancin is provided in dry powder (*e.g.*, lyophilized) form and reconstituted in a pharmaceutically acceptable carrier, such as a sterile aqueous formulation, prior to administration to an individual. In one embodiment, the pharmaceutically acceptable carrier includes 5% dextrose in water. A pharmaceutical composition of the invention may be administered to a mammal in need of treatment or prevention of a bacterial infection, such as a human. In some embodiments, a pharmaceutical composition may include at least one antibiotic that is not dalbavancin, such as an antibiotic that is effective (*e.g.*, bactericidal) against a Gram-negative bacterium and/or an antibiotic that is effective against Gram-positive species against which dalbavancin is not effective, such as VanA vancomycin-resistant bacterial strains.

[0015] One or more stabilizing substances are employed to inhibit degradation of one or more dalbavancin components during storage as a dry powder (*e.g.*, lyophilized) formulation and/or as an aqueous formulation prior to administration to an individual. Over time, degradation can result in the undesirable formation of less active and/or inactive components which could potentially cause adverse effects *in*

vivo. Preferred stabilizers include nonionic components such as sugars or sugar alcohols, *e.g.*, a mono-, di-, or polysaccharide, or derivative thereof, such as, for example, mannitol, lactose, sucrose, sorbitol, glycerol, cellulose, trehalose, maltose, or dextrose, or mixtures thereof.

[0016] In another aspect, methods are provided for treating a bacterial infection in an individual in need thereof, including administering at least one unit dose of dalbavancin in an amount sufficient to provide a therapeutically effective plasma level of dalbavancin in the individual for at least five days, and a pharmaceutically acceptable carrier. A therapeutically effective plasma level of dalbavancin is generally at least about 4 mg of dalbavancin per liter of plasma. In one embodiment, the dosage amount of dalbavancin administered is an amount that is clinically effective and also has reduced adverse side effects in comparison to the standard of care with drugs such as teicoplanin and vancomycin.

[0017] Dalbavancin may be administered as a single dose or as multiple doses. In some embodiments, a single dose of about 100 mg to about 4000 mg, for example 3000 mg, of dalbavancin is administered. In various embodiments, a single dalbavancin dose may include at least about any of 0.1, 0.25, 0.5, 1, 1.5, 2, 2.5, or 3 grams.

[0018] In other embodiments, two doses are administered about five to about ten days apart, such as about one week apart. The first dose may be about 500 to about 5000 mg of dalbavancin and the second dose may be about 250 mg to about 2500 mg of dalbavancin. Often, the first dose includes about 1.5 to about 3 times, often at least about twice as much of the amount of dalbavancin contained in the second dose. For example, the first dose may be about 1000 mg and the second dose may be about 500 mg of dalbavancin. In methods in which two doses are administered, the plasma trough level of dalbavancin in an individual prior to administration of the second dose is generally at least about 4 mg, often at least about 10 mg, often at least about 20 mg, more often at least about 30 mg dalbavancin per liter of plasma, and still more often at least about 40 mg dalbavancin per liter of plasma.

[0019] Often, methods of the invention include parenteral administration, for example intravenous administration. In some embodiments, administration is intravenous with the rate of administration controlled such that administration occurs over at least about 30 minutes or longer.

[0020] Methods of the invention may be used to treat a Gram-positive bacterial infection, such as, for example, a *Staphylococcus aureus* or *Streptococcus pyogenes* skin and soft tissue infection. In some embodiments, the infection is penicillin-resistant and/or multi-drug resistant.

[0021] In another aspect, a method for preventing a bacterial infection is provided which includes administering at least one unit dose of dalbavancin in an amount sufficient to provide a prophylactically effective plasma level of dalbavancin in the individual for at least about one day, three days, five days, one week, or ten days or longer, and a pharmaceutically acceptable carrier. The dosage of dalbavancin may be, for example, about 100 mg to about 1000 mg. In some embodiments, dalbavancin is administered prior, during, or subsequent to a medical procedure or a stay in the hospital.

[0022] Therapeutic or prophylactic methods of the invention may include administration of at least one antibiotic that is not dalbavancin, preferably an antibiotic that is effective against a Gram-negative bacterium and/or an antibiotic that is effective against Gram-positive strains that dalbavancin is not effective against, such as VanA strains.

[0023] In another aspect, kits are provided that include at least one unit dose of dalbavancin in an amount sufficient to provide a therapeutically effective plasma level for at least about five days or a prophylactically effective plasma level of dalbavancin for at least about one day in an individual, and instructions for use in a method of treatment or prophylaxis of a bacterial infection. A kit may contain two unit dosages, with a first dosage including 1.5 to 3 times, often at least about twice as much of the amount of dalbavancin included in a second dosage. Kits may also

include an antibiotic that is not dalbavancin, preferably effective against a Gram-negative bacterium.

[0024] In one embodiment, kits are provided that include a first container containing a dry powder (*e.g.*, lyophilized) dalbavancin composition and a second container containing a predetermined amount of a physiologically acceptable aqueous solution for admixing with the dalbavancin composition. Such solutions are preferably sterile aqueous solutions. In one embodiment, kits include a delivery means for administering the dalbavancin composition to an individual, for example a syringe or intravenous administration means.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **Figure 1** depicts dalbavancin plasma concentration versus time following a single 1000 mg intravenous infusion of dalbavancin.

[0026] **Figure 2** depicts isothermal titration calorimetry data for dalbavancin binding to human serum albumin (top) and a graphical representation of the data fitted to a curve determined from a 2:1 binding model of dalbavancin:protein (bottom).

[0027] **Figure 3** depicts an electrospray ionization mass spectrum of dalbavancin.

[0028] **Figure 4** is a graph of dalbavancin concentration vs. population ratio of dalbavancin multimer to monomer and depicts an increase in population ratio of dalbavancin multimer to monomer with increasing dalbavancin concentration.

[0029] **Figure 5** is a graph of pH vs. population ratio of dalbavancin multimer to monomer and depicts an increase in population ratio of dalbavancin multimer to monomer with increasing pH.

[0030] **Figure 6** depicts an electrospray ionization mass spectrum of dalbavancin in an ammonium formate 5mM pH 5 solution.

[0031] **Figure 7** depicts an electrospray ionization mass spectrum of dalbavancin in an ammonium formate 50 mM pH 5 solution.

[0032] **Figure 8** depicts an electrospray ionization mass spectrum of dalbavancin in an ammonium formate 100 mM pH 5 solution.

[0033] **Figure 9** depicts an electrospray ionization mass spectrum of teicoplanin (50 $\mu\text{g/mL}$) in water.

[0034] **Figure 10** depicts an electrospray ionization mass spectrum of teicoplanin (100 $\mu\text{g/mL}$) in water.

[0035] **Figure 11** depicts the effect of HSA on the apparent dissociation constant for dalbavancin/tri-peptide binding at 26°C (pH 7.4).

[0036] **Figure 12** depicts a comparison of isothermal calorimetry (ITC) data for binding of tri-peptide to vancomycin and dalbavancin under identical conditions, using the same tri-peptide solution.

[0037] **Figures 13A and 13B** depict the possible interaction of dalbavancin monomers and multimers (including dimers) with tri-peptide ligand and HSA.

Figure 13A depicts dalbavancin in monomer-dimer equilibrium in solution, binding as monomer to two separate sites on HSA. **Figure 13B** depicts ligand binding to dalbavancin dimer in solution and more weakly to dalbavancin monomers attached to HSA.

DETAILED DESCRIPTION OF THE INVENTION

[0038] The present invention provides improved dosage regimes and novel compositions of dalbavancin, and improved methods of treatment of antibiotic-resistant bacterial infections. In particular, the invention provides dalbavancin compositions having activity against one or more antibiotic resistant strains of bacteria, such as MRSA, which may be administered in a dosing regimen of once every 5-7 days or longer.

[0039] Dalbavancin, which is also referred to in the scientific literature as BI 397 or VER001, is a semi-synthetic glycopeptide mixture, the properties of which have been reported in U.S. Pat. Nos. 5,606,036, 5,750,509, 5,843,679, and 5,935,238.

[0040] As used herein, the term “dalbavancin” refers to compositions comprising one or more, preferably two or more, closely related homologs, termed “A₀,” “A₁,” “B₀,” “B₁,” “C₀,” and “C₁,” as described below, or monomers, multimers (*i.e.*, dimer or higher order multimer), tautomers, esters, solvates, or pharmaceutically acceptable salts thereof. As used herein, “dimer” or “multimer” refers to either a homodimer or homomultimer, *i.e.*, a dimer or multimer composed of monomers of the same dalbavancin homolog, or a heterodimer or heteromultimer, *i.e.*, a dimer or multimer composed of monomers of at least two different dalbavancin homologs. Dalbavancin often includes “MAG,” a non-homolog variant described below. Individually, dalbavancin homologs and MAG are sometimes referred to herein as “dalbavancin components.”

[0041] Dalbavancin is prepared by chemical modification of the natural glycopeptide complex A-40,926 as described in Malabarba and Donadio (1999) *Drugs of the Future* 24(8):839-846. The predominant component of dalbavancin is Factor B₀, which accounts for >75% of the whole complex.

[0042] The amount of each of the components present in a dalbavancin composition is dictated by a variety of factors, including, for example, the fermentation conditions employed in the preparation of the natural glycopeptide complex A-40926, which is the precursor to dalbavancin (see, *e.g.*, U.S. Pat. No. 5,843,679), the conditions employed to recover A-40926 from the fermentation broth, the chemical reactions employed to selectively esterify the carboxyl group of the sugar moiety of A-40926, the conditions employed to amidate the peptidyl carboxyl group, the conditions employed to saponify the ester of the carboxyl group of the N-acylaminoglucuronic acid function, the conditions employed to recover dalbavancin from the synthetic mixture, and the like.

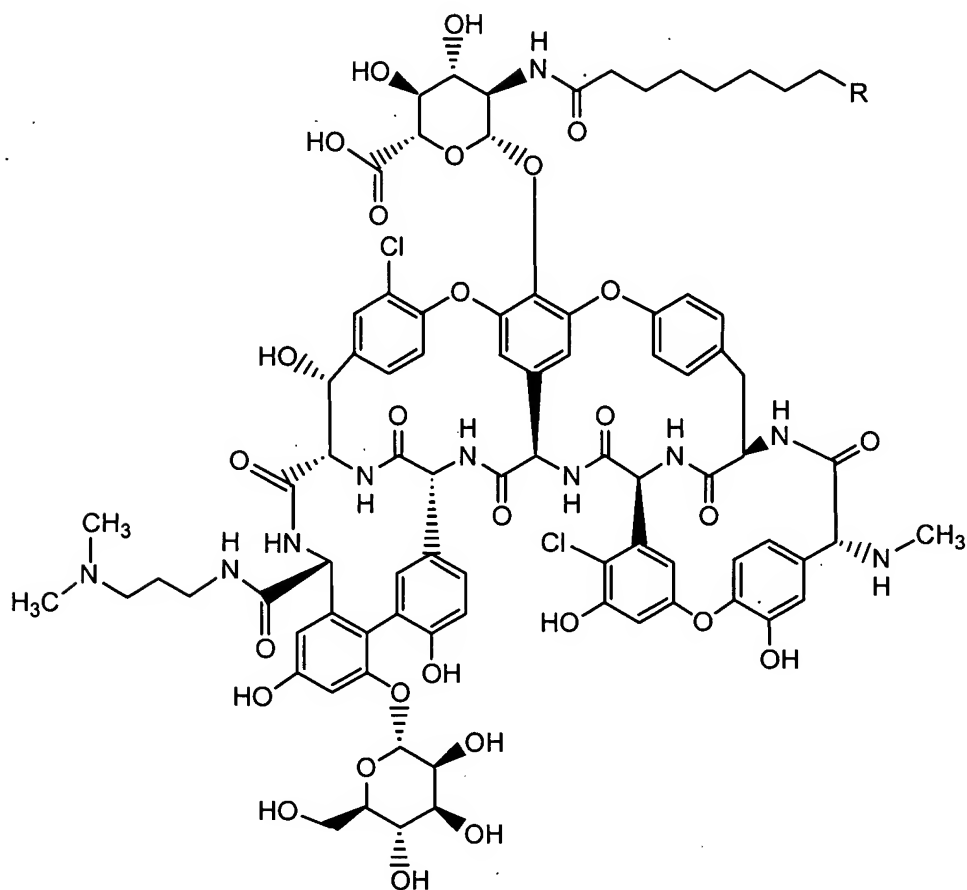
[0043] In preferred embodiments, dalbavancin compositions comprise at least about 80 to about 98% by weight of the B₀ component. In particularly preferred embodiments, dalbavancin comprises the following amounts of B₀:

Table 1. Preferred Amounts of B₀ Component in Dalbavancin Composition

Preferred¹	More Preferred¹	Even More Preferred¹
80-98	80-97	80-96
81-98	81-97	81-96
82-98	82-97	82-96
83-98	83-97	83-96
84-98	84-97	84-96
85-98	85-97	85-96
86-98	86-97	86-96
87-98	87-97	87-96
88-98	88-97	88-96
89-98	89-97	89-96
90-98	90-97	90-96

- 1** each range represents the mole % of B₀ relative to the total dalbavancin components present in the dalbavancin composition including MAG

[0044] The chemical structure of several of the dalbavancin components is depicted in Formula I below:



I

Dalbavancin Component	R	Molecular Weight
A ₀	-CH(CH ₃) ₂	1802.7
A ₁	-CH ₂ CH ₂ CH ₃	1802.7
B ₀	-CH ₂ CH(CH ₃) ₂	1816.7
B ₁	-CH ₂ CH ₂ CH ₂ CH ₃	1816.7
C ₀	-CH ₂ CH ₂ CH(CH ₃) ₂	1830.7
C ₁	-CH ₂ CH ₂ CH ₂ CH ₂ CH ₃	1830.7

[0045] All of the above dalbavancin components are bactericidally active against a number of Gram-positive bacteria. However, one non-homologous dalbavancin component, termed "MAG," which lacks an acylglucoronamine moiety present in other components, is less bactericidally effective, both *in vivo* and *in vitro*, than other dalbavancin components. MAG is thought to be a decomposition product of one or more of the other dalbavancin components. Accordingly, in a preferred embodiment, the amount of MAG in dalbavancin is less than about 4, 3.5, 3, 2.5, 2, 1.5, 1, or 0.5 mole percent of all dalbavancin components present, including MAG.

[0046] Dalbavancin is thought to inhibit the biosynthesis of the bacterial cell wall by binding to D-alanyl-D-alanine-terminating precursors of peptidoglycans. Dimeric or higher order multimers of dalbavancin may possess further antibacterial properties by interaction of the lipophilic side chains with the cytoplasmic membrane of bacteria. See, for example, Malabarba and Ciabatti, et al. (2001) *Current Medicinal Chemistry* 8:1759-1773. A further elaboration on dalbavancin multimers may be found in U.S. Serial No. 10/____, entitled "DALBAVANCIN COMPOSITIONS FOR TREATMENT OF BACTERIAL INFECTIONS," filed on November 14, 2003, as Attorney Docket No. 34231-20052.00, the disclosure of which is hereby incorporated by reference in its entirety.

[0047] *In vitro*, nonclinical, and clinical data indicate dalbavancin to be of benefit for the treatment of serious Gram-positive infections caused by MRSA and CoNS, and all streptococcal and non-VanA enterococcal species, including VanB and VanC phenotypes poorly susceptible or resistant to vancomycin.

[0048] Dalbavancin is more active *in vitro* against staphylococci (including some teicoplanin-resistant strains) than teicoplanin and vancomycin. Dalbavancin has better activity against streptococci, including penicillin-resistant strains, than teicoplanin or vancomycin. Dalbavancin is active *in vitro* and *in vivo* against a number of Gram-positive bacteria, including most drug resistant strains.

[0049] Dalbavancin is typically administered to an individual as a dalbavancin composition. As used herein, the term “dalbavancin composition” or “dalbavancin formulation” refers to a composition, typically a pharmaceutical composition comprising dalbavancin, as defined above, and one or more other non-dalbavancin components such as, for example, a pharmaceutically acceptable carrier, a stabilizer, a buffers, or other similar components.

[0050] As shown in Example 1, dalbavancin is effective at dose intervals of one week. Thus, an advantage of dalbavancin versus other treatment options is the ability to administer this antibiotic on a once-weekly basis, thereby maximizing patient compliance and potentially minimizing the need for or decreasing the length of a hospital stay for parenteral antibiotic administration. Less frequent dosing often permits treatment on an out-patient basis, thus decreasing treatment costs. As further shown in Example 1, a second dose of dalbavancin approximately one week after administration of the first dosage, where the second dose is approximately one-half the first dose, unexpectedly provides significant improvement in the efficacy of treatment.

Methods of use

[0051] Methods are provided for administration of dalbavancin to an individual in need of treatment for a bacterial infection. Treatment can include prophylaxis, therapy, or cure. Methods include administration of one or more unit doses of dalbavancin in a therapeutically or prophylactically effective amount.

[0052] As used herein, “therapeutically effective amount” refers to the amount of dalbavancin that will render a desired therapeutic outcome (*e.g.*, reduction or elimination of a bacterial infection). A therapeutically effective amount may be administered in one or more doses. A “prophylactically effective amount” refers to an amount of dalbavancin sufficient to prevent or reduce severity of a future bacterial infection when administered to an individual who is susceptible to and/or who may contract a bacterial infection, *e.g.*, by virtue of a medical procedure or stay in the

hospital, or exposure to an individual with a bacterial infection. Dalbavancin is generally administered in a pharmaceutically acceptable carrier.

[0053] Dalbavancin is often provided as a hydrochloride salt, which is freely soluble in water.

[0054] Typically, dalbavancin is administered as a “unit dose” in a dalbavancin formulation which includes an amount of dalbavancin sufficient to provide a therapeutically or prophylactically effective plasma level of dalbavancin for several days, often at least about 5 days, one week, or 10 days, when administered to an individual.

[0055] As used herein, “individual” refers to a vertebrate, typically a mammal, often a human.

[0056] All homologs of dalbavancin described above exhibit a prolonged half-life in plasma, often 9 days or more, although MAG is thought to have a shorter half-life than other homologs. The long half-life permits longer intervals between dosages than vancomycin or teicoplanin. As described in Example 1, weekly dosing of dalbavancin is effective for control of bacterial infections, in contrast to the twice daily dosing schedule which is often used for vancomycin or the once daily schedule generally used for teicoplanin. Less frequent dosing of dalbavancin offers significant treatment advantages over vancomycin and teicoplanin, particularly with regard to improved convenience and patient compliance with the treatment regimen.

Surprisingly high doses (*i.e.*, resulting in surprising high and long-lasting serum levels) can be administered, and with less frequency than other available treatment options. The novel dosage regimen available for dalbavancin results in improved efficacy because at concentrations required to effect less frequent dosing, dalbavancin exhibits minimal adverse effects *in vivo*, evidencing a large pharmaceutical window, and further because blood levels of dalbavancin are maintained above minimum bactericidal levels for the entire treatment protocol, evidencing a prolonged serum half-life for dalbavancin. The combination of the large pharmaceutical window coupled with prolonged serum half-life permits less frequent dosing of dalbavancin.

[0057] In addition, dalbavancin is preferably formulated with a stabilizer which inhibits degradation of one or more of the components of dalbavancin. In one preferred embodiment, dalbavancin is formulated with a 1:2 weight ratio of mannitol:dalbavancin. In another preferred embodiment, dalbavancin is formulated with a 1:1:4 weight ratio of mannitol:lactose:dalbavancin.

[0058] In some embodiments, a dalbavancin formulation is administered at a dosage that results in therapeutically effective (*i.e.*, bactericidal) plasma levels of the drug for several days, often at least about 5 to about 10 days, often at least about one week. Generally, dalbavancin is maintained in plasma at or above the minimum bactericidal concentration of about 4 mg/l for at least 5 days. Often, dalbavancin is maintained at a plasma level of at least about 5 mg/l, often at least about 10 mg/l, often at least about 20 mg/l, often at least about 30 mg/l, often at least about 40 mg/l, for at least 5 days, often at least about one week or longer. Plasma levels of dalbavancin may be measured by methods that are well known in the art, such as liquid chromatography, mass spectrometry, or microbiological bioassay. An example of a method for quantitating dalbavancin in plasma is provided in Example 5.

[0059] Upper limits for dalbavancin plasma concentration levels are generally dictated by dosages which inhibit unacceptable adverse effects in the patient population treated.

[0060] Dalbavancin compositions may be administered in a single dose or in multiple doses. When administered as a single dose, the dalbavancin composition is preferably formulated to contain sufficient amounts of dalbavancin to effect antibacterial properties *in vivo* for at least 5 days, preferably at least 7 days, and more preferably at least 10 days.

[0061] When multiple doses are employed, dalbavancin can be administered weekly for two or more weeks. In one embodiment, dalbavancin is administered in at least two doses, often in two doses about 5 to about 10 days apart, more often once a

week for two weeks. As shown in Example 1, such a dosing regimen provides significant advantages over conventional antibiotic treatment protocols.

[0062] Dalbavancin compositions also may be administered in multiple doses two or more days or at least one week apart or in one or more biweekly doses. In some embodiments, a dalbavancin composition is administered weekly, followed by biweekly, or monthly administration. In some embodiments, dalbavancin is administered at weekly intervals for 2, 3, 4, 5, 6, or more weeks.

[0063] Most advantageously, daily dosing is not required because higher, less frequent doses are used. Single or multiple doses may range, for example, from about 0.1 to about 5 grams. A single dose of about 0.1 to about 4 grams, *e.g.*, about 3 grams, may be administered for various infection treatments. Where multiple doses are administered, for example, weekly, each dose may range, for example, from about 0.25 to about 5.0 grams.

[0064] For embodiments in which a single dose is administered to treat an infection, the amount of the dose may be, for example, about 0.1 to about 5 grams, or about 0.5 to about 4 grams, or about 1 to about 3.5 grams, or about 2 to about 3 grams *e.g.*, about 3 grams. In some embodiments, a single dose of about 1, 1.5, 2, 2.5, or 3 grams is administered for treatment of a bacterial infection. For embodiments in which a single dose is administered for prophylaxis, the amount of the dose may be, for example, about 0.1 to about 3 grams, or about 0.1 to about 1 gram, *e.g.*, about 0.5 or about 0.25 gram.

[0065] In dosing schemes that include multiple dosages, the individual dosages may be the same or different. In some embodiments, a first, higher dose is administered, that is, for example, about 1.5 to 3 times higher, than one or more subsequent doses. For example, the first dose may be about 0.5 grams to about 5 grams and the second dose about 0.25 grams to about 2.5 grams, the first dose may be about 0.8 to about 2 g and the second dose about 0.4 to about 1 gram, or the first dose may be about 0.4 to about 3 g and the second dose about 0.2 to 1.5 g.

[0066] In some embodiments, at least two dosages are administered wherein the first dosage includes about twice as much dalbavancin as subsequent dosages. In one embodiment, a first dosage includes about 1 gram of dalbavancin and a subsequent dosage includes about 0.5 gram. In another embodiment, a first dosage includes about 0.5 gram of dalbavancin and a subsequent dosage includes about 0.25 gram.

[0067] In some embodiments, a dalbavancin composition is administered in two doses of equal or different amount two or more days or at least about one week apart. Often, two doses of about 0.2 to about 1.5 grams of dalbavancin are administered about 5 to about 10 days apart, more often about 1 week apart. In one embodiment, a first dosage of about 1 gram of dalbavancin and a second dosage of about 0.5 gram of dalbavancin are administered about 1 week apart.

[0068] In a multiple dosing regimen, the time between doses may range, for example, from about 5 to about 10 days, often about one week. Dose frequency may be, for example, two weekly doses, or multiple weekly doses. The dosing interval, or time between doses, can be, for example, any of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more days. The number of doses given, can be, for example, one, two, three, four, five, six or more doses, each dose after the initial dose being given after the selected dosage interval.

[0069] In a multiple dosing scheme, often the “trough level,” or the level of dalbavancin in plasma after a first dose of dalbavancin and just prior to administration of a second dose, is at least about 4 mg/l. Preferably, the trough level at the end of a dosing interval such as about one week is at least about 20 mg/l, more preferably at least about 30 mg/l, and even more preferably at least about 40 mg/l.

[0070] Dalbavancin can be administered parenterally, *e.g.*, intramuscularly (i.m.), intravenously (i.v.), subcutaneously (s.c.), intraperitoneally (i.p.), or intrathecally (i.t.). The dosing schedule and actual dosage administered may vary depending on such factors as the nature and severity of the infection, the age, weight, and general health of the patient and the tolerance of a particular patient to dalbavancin, but will

be ascertainable to health professionals. In one embodiment, a one gram intravenous dose of dalbavancin is followed by a 0.5 gram intravenous dose one week later.

[0071] Administration and delivery of the drug to the patient, *e.g.*, intravenously, can be done at a controlled rate, so that the concentration in the blood does not increase too quickly or cause precipitation to occur. In some embodiments, dalbavancin is administered at an appropriate rate such that the drug forms a complex with endogenous protein(s) in the bloodstream. Without intending to be bound to a particular theory, it is believed that endogenous protein, such as human serum albumin, can form a complex *in vivo* with one or two molecules of dalbavancin homolog monomers. When a sufficient amount of dalbavancin is present, it is believed that up to two molecules of dalbavancin homolog will bind to the endogenous protein and it is further believed that this complex is formed by binding of separate homolog molecules of dalbavancin at two different binding sites. Alternatively, it is possible that dimeric dalbavancin is binding to a single binding site on the endogenous protein. A further elaboration on the dalbavancin-endogenous protein complexes discussed above may be found in U.S. Serial No. 10/____, entitled "COMPOSITIONS AND METHODS FOR TREATING BACTERIAL INFECTIONS WITH PROTEIN-DALBAVANCIN COMPLEXES," filed on November 14, 2003, as Attorney Docket No. 34231-20053.00, the disclosure of which is hereby incorporated by reference in its entirety.

[0072] The infusion duration can be, for example, about 1 minute to about 2 hours. For example, an infusion duration of about 30 minutes may be used where the dose is about 0.5 to about 1 gram. Intravenous administration under controlled rate conditions can generate concentrations of dalbavancin in the body that are in great excess of what can be achieved in the solution phase at physiological pH *in vitro*. Although not wishing to be limited by theory, this may be due to the formation of a complex of dalbavancin with endogenous protein(s) such as serum albumin, which may increase the capacity of plasma to absorb dalbavancin.

[0073] Formation of a dalbavancin complex *in vitro* or *ex vivo* may permit faster administration, such as at least about 1 minute, at least about 10 minutes or at least about 20 minutes. Such a complex can be achieved by mixing human serum albumin and/or another endogenous protein with dalbavancin, thereby forming the complex *in vitro* or *ex vivo*, and then administering this complex to the treated patient.

Alternatively, the human serum albumin or other endogenous protein may be obtained from autologous sources or by expression from a microorganism modified to contain the gene for the protein.

[0074] The amount of dalbavancin administered may be any of the dosages disclosed herein. The dalbavancin dose is generally chosen such that the drug will remain at a therapeutically or prophylactically effective (*i.e.*, bactericidal) plasma level for an extended period of time, often at least 5 days, more often about one week or longer. Administration of a dose of dalbavancin which produces and maintains bactericidal concentrations for at least about one week (or about 5 to about 10 days) is preferred. A bactericidal concentration is defined as the concentration of dalbavancin required to kill at least 99% of the bacteria present at the initiation of an *in vitro* experiment over a 24 hour period. A minimum bactericidal concentration of dalbavancin in plasma is typically about 4 mg/l.

[0075] Examples of indications that can be treated include both complicated and uncomplicated skin and soft tissue infections (SSTI), blood stream infections (BSI), catheter-related blood stream infections (CRBSI), osteomyelitis, prosthetic joint infections, surgical prophylaxis, endocarditis, hospital or community acquired pneumonia, pneumococcal pneumonia, empiric treatment of febrile neutropenia, joint space infections, and device infections (*e.g.*, pace makers and internal cardiac defibrillators). Gram-positive or antibiotic-resistant bacterial infections may be treated, such as a *Staphylococcus*, *Streptococcus*, *Neisseria*, or *Clostridium* genus infection, in particular *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hemolyticus*, *Streptococcus pyogenes*, Groups A and C *Streptococcus*, *Neisseria gonorrhoeae*, or *Clostridium difficile*.

[0076] The invention provides methods for treatment of skin and soft tissue infections (SSTIs). Patients who may benefit from this treatment may have either deep or superficial infections. SSTI may involve deeper soft tissue and/or require significant surgical intervention, such as for example a major abscess, infected ulcer, major burn, or deep and extensive cellulitis. Infected surgical wounds may also be treated.

[0077] The clinical presentation of skin and skin structure infection may vary from mild folliculitis to severe necrotizing fasciitis. The mode of acquisition may also vary with community-acquired skin and skin structure infections, which are often preceded by injuries resulting from occupational exposure or recreational activities, and are usually associated with a greater diversity of pathogens. Hospital-acquired skin and skin structure infections are generally associated with surgical procedures, the development of pressure sores, and catheterization. Post-surgical infections are the third most frequent nosocomial infection and account for 17% of all nosocomial infections reported to the National Nosocomial Infection Surveillance System (NNIS). The most frequent source of infection is the patient's endogenous flora. *Staphylococcus aureus*, coagulase-negative staphylococci, and *Enterococcus spp.* are the pathogens most frequently isolated from SSTIs.

[0078] Symptoms of SSTI infections may include erythema, tenderness or pain, heat or localized warmth, drainage or discharge, swelling or induration, redness, or fluctuance. Patients that may benefit from treatment with the methods of the invention include those with deep or complicated infections or infections that require surgical intervention, or patients with underlying diabetes mellitus or peripheral vascular disease. These infections are often caused by Gram-positive bacteria such as *Staphylococcus* or *Streptococcus* species, such as *Staphylococcus aureus* or *Streptococcus pyogenes*. Methods for treatment of a skin or soft tissue bacterial infection include administering a therapeutically effective amount of dalbavancin to an individual in need of treatment, in an amount and according to a dosing regime as discussed above. In some embodiments, a dalbavancin composition is administered

intravenously in two doses, often about 5 to about 10 days apart, more often about 1 week apart. In some embodiments, the first dosage includes at least twice as much dalbavancin as the second dosage. In one embodiment, the first dosage is about 1000 mg and the second dosage is about 500 mg.

[0079] The invention also provides methods for prophylactic prevention of the onset of a bacterial infection, for example an infection caused by *Staphylococcus aureus*, or by a *Neisseria* or *Clostridium* genus bacterium. In a prophylactic method of the invention, a prophylactically effective amount of dalbavancin is administered to an individual who may be susceptible to contracting a bacterial infection, for example, through a medical procedure. Often, dalbavancin is administered in an amount sufficient to provide a prophylactically effective plasma level for at least about 1 day, at least about 3 days, at least about 5 days, or at least about one week or longer. Dalbavancin compositions may be administered, for example, parenterally, *e.g.*, via intramuscular (i.m.), intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.), or intrathecal (i.t.) injection, prior or subsequent to surgery as a preventative step against infection. Dalbavancin compositions may be administered immediately prior or subsequently to, 1 or more days or about one week prior or subsequently to, or during an invasive medical procedure such as surgery or a stay in a medical care facility such as a hospital to prevent infection. A prophylactic method may be used in any situation in which it is possible or likely that an individual may contract a bacterial infection, including situations in which an individual has been exposed to or is likely to be exposed to a bacterially infected individual. For prophylactic methods, dalbavancin compositions may be administered as either a single dose or as two or more doses of equal or different amount that are administered several days to about one week apart. In one embodiment, a dalbavancin composition may be administered prior to or simultaneously with insertion of an intravenous catheter in order to prevent a bloodstream related infection.

[0080] For prophylactic methods, dalbavancin compositions may be administered in a single dose or in multiple doses, according to any of the dosing schemes

described above. Often, a dalbavancin composition is administered as a single dose comprising about 0.1 to about 3 grams, or about 0.1 to about 1 gram, *e.g.*, about 0.25 gram or about 0.5 gram. In one embodiment, a single dose of about 0.25 gram is administered intravenously over a time frame of about 2 minutes to about 1 hour, *e.g.*, about 30 minutes. In another embodiment, the dalbavancin composition is administered intravenously simultaneously with administration of another pharmaceutical (*e.g.*, antibiotic) treatment.

[0081] In any of the therapeutic or prophylactic methods described above, the dalbavancin composition may be administered either simultaneously or sequentially with at least one other antibiotic. In some embodiments, at least one other antibiotic that is effective (*e.g.*, bactericidal) against one or more Gram-negative bacterial species and/or a Gram-positive bacterial strain against which dalbavancin is not effective is administered in addition to dalbavancin. In some embodiments, dalbavancin and at least one antibiotic that is effective (*e.g.*, bactericidal) against at least one Gram-negative bacterial species is administered as a mixture in the dalbavancin composition.

Pharmaceutical compositions

[0082] The invention provides pharmaceutical compositions formulated for administration of dalbavancin according to the methods described above. Pharmaceutical compositions of the invention may be in the form of a unit dose of dalbavancin that includes an amount of dalbavancin sufficient to provide a therapeutically or prophylactically effective plasma level of dalbavancin for several days, often at least about 3 days, at least about 5 days, or at least about one week or longer when the composition is administered to an individual, and a pharmaceutically acceptable carrier. Generally, a therapeutically or prophylactically effective plasma level of dalbavancin is at least about 4 mg per liter of plasma. Plasma levels of dalbavancin may be measured by well known methods in the art, such as those described above.

[0083] Dalbavancin may optionally be in a pharmaceutically acceptable form for administration to an individual, optionally as a pharmaceutically acceptable, non-toxic salt.

[0084] Examples of suitable salts of dalbavancin include salts formed by standard reaction with both organic and inorganic acids such as, for example, hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, trifluoroacetic, trichloroacetic, succinic, citric, ascorbic, lactic, maleic, glutamic, camphoric, glutaric, glycolic, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic, and the like acids. Representative examples of bases that can form salts with dalbavancin include alkali metal or alkaline earth metal hydroxides such as sodium, potassium, calcium, magnesium, and barium hydroxide, ammonia and aliphatic, alicyclic, or aromatic organic amines such as methylamine, dimethylamine, diethylamine, ethanolamine, and picoline. (See, for example, U.S. Pat. No. 5,606,036.)

[0085] In some embodiments, a pharmaceutically acceptable aqueous formulation of dalbavancin is provided that is suitable for parenteral administration, such as, for example, intravenous injection. For preparing such an aqueous formulation, methods well known in the art may be used, and any pharmaceutically acceptable carriers, diluents, excipients, or other additives normally used in the art may be used. In one embodiment, a pharmaceutically acceptable aqueous formulation for intravenous injection includes 5% dextrose.

[0086] A pharmaceutical composition for parenteral administration includes dalbavancin and a physiologically acceptable diluent such as deionized water, physiological saline, 5% dextrose, water miscible solvent (*e.g.*, ethyl alcohol, polyethylene glycol, propylene glycol, etc.), non-aqueous vehicle (*e.g.*, oil such as corn oil, cottonseed oil, peanut oil, and sesame oil), or other commonly used diluent. The formulation may additionally include a solubilizing agent such as polyethylene glycol, polypropylene glycol, or other known solubilizing agent, buffers for stabilizing the solution (*e.g.*, citrates, acetates, and phosphates) and/or antioxidants

(*e.g.*, ascorbic acid or sodium bisulfite). (See, for example, U.S. Patent No. 6,143,739.) Other suitable pharmaceutical carriers and their formulations are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. As is known in the art, pharmaceutical preparations of the invention may also be prepared to contain acceptable levels of particulates (*e.g.*, particle-free) and to be non-pyrogenic (*e.g.*, meeting the requirements of an injectable in the U.S. Pharmacopeia).

[0087] In one embodiment, a pharmaceutical composition is provided by dissolving a dried (*e.g.*, lyophilized) dose of dalbavancin, often containing a stabilizer or mixture of stabilizers, in an amount of water and preferably deionized water in a volume sufficient for solubilization. Typically, the amount of water sufficient for solubilization is approximately 10 mL and the resulting pH of the dalbavancin solution is above 3.0, and about 3.5 to 4.5. Diluting this solution by adding it to a second amount of an aqueous diluent, often containing 5% dextrose, such as an amount contained in a drip bag for intravenous administration, raises the pH of the dalbavancin solution to about 5 to 5.5. In another embodiment, the pH of the dalbavancin solution in a drip bag is about 4.5. The second amount of aqueous solution may be deionized or sterile, or both deionized and sterile. In one embodiment, the aqueous diluent is 5% dextrose.

[0088] Pharmaceutical compositions for parenteral administration may be made up in sterile vials containing one or more unit doses of dalbavancin in a therapeutically or prophylactically effective amount as described above, optionally including an excipient, under conditions in which bactericidal effectiveness of dalbavancin is retained. The composition may be in the form of a dry (*e.g.*, lyophilized) powder. Prior to use, a physiologically acceptable diluent may be added and the solution withdrawn via syringe for administration to a patient. A pharmaceutical formulation as described above may be sterilized by any acceptable means including, for example, e-beam or gamma sterilization methods, or by sterile filtration.

[0089] A typical formulation for parenteral administration may include dalbavancin at a concentration such as about 0.1 to about 100 mg, about 0.5 to about 50 mg, about 1 to about 10 mg, or about 2 to about 4 mg of dalbavancin per ml of final preparation.

[0090] In some embodiments, a pharmaceutical composition in accordance with the invention includes a mixture of dalbavancin and one or more additional antibiotics.

Preferably, at least one non-dalbavancin antibiotic in the mixture is effective (*e.g.*, bactericidal) against one or more species of Gram-negative bacteria, such as, for example, azthreonam, and/or against one or more Gram-positive bacterial strains against which dalbavancin is not effective, such as, for example, ilnezolide or daptomycin. The mixture may also include a pharmaceutically acceptable carrier as described above.

[0091] In some embodiments, pharmaceutical compositions of the invention include one or more stabilizing substances which inhibit degradation of one or more of the components of dalbavancin to less active or inactive materials, for example, MAG. As used herein, “stabilizing substance” or “stabilizer” refers to a substance that stabilizes the level of one or more of the constituent components of dalbavancin, for example, B₀, in the composition. A “stabilizing effective amount” refers to an amount of a stabilizer sufficient to enhance long-term stability of one or more components of a dalbavancin composition. In some embodiments, a stabilizing effective amount may be provided by a mixture of two or more stabilizing substances, each of which alone is not present in an amount sufficient to provide a stabilizing effect.

[0092] Examples of stabilizers include, for example, nonionic substances such as sugars, *e.g.*, mono-, di-, or polysaccharides, or derivatives thereof, sugar alcohols, or polyols. Such stabilizing substances include, for example, mannitol, lactose, sucrose, sorbitol, glycerol, cellulose, trehalose, maltose, raffinose, or mixtures thereof.

[0093] In one embodiment, the pharmaceutical composition includes a weight ratio of 1:2 mannitol:dalbavancin. In another embodiment, the pharmaceutical composition includes a weight ratio of 1:1:4 mannitol:lactose:dalbavancin.

Surprisingly, it has been found that a combination of mannitol and lactose provides a greater stabilizing effect than either substance alone. Often, the pH of a pharmaceutical composition of the invention is, for example, about 3 to about 5, for example about 3.5 or about 4.5.

[0094] In some embodiments, one or more procedures may be employed to reduce formation of MAG. For example, freeze drying of dalbavancin in the presence of a stabilizing substance, such as mannitol, may be employed to reduce the amount of MAG formed.

[0095] Storage of dalbavancin compositions is often at lower than ambient temperature, such as at about 5° C, to enhance stability.

Improved efficacy and reduced side effects

[0096] Weekly dosing of dalbavancin at high dosage levels (*i.e.*, resulting in surprising high and long-lasting serum levels) shows a surprisingly good safety profile, similar to, or better than, that observed with the standard therapy of lower doses of conventional antibiotics administered daily or even 2-4 times daily, as demonstrated by the Examples herein. A surprisingly high dosage (*i.e.*, resulting in surprising high and long-lasting serum levels) of dalbavancin may be administered, with less frequency than other antibiotics, and without adverse side effects, enabling improved efficacy and patient compliance.

[0097] As discussed in Example 1, treatment with dalbavancin results in a low incidence of adverse events. Serious adverse events include any adverse drug experience occurring at any dose that results in death, is life-threatening, results in hospitalization or prolongation of existing hospitalization, or persistent or significant disability or incapacity. In the Phase II trial described in Example 1, 90% of adverse reactions, such as diarrhea, nausea, hyperglycemia, limb pain, vomiting, and constipation, were mild to moderate in severity. Use of dalbavancin in the trial in Example 1 resulted in no serious adverse events related to study drug treatment.

Kits

[0098] The invention also provides kits for use in methods of treatment or prophylaxis of bacterial infections. The kits include a pharmaceutical composition of the invention, for example including at least one unit dose of dalbavancin, and instructions providing information to a health care provider regarding usage for treating or preventing a bacterial infection. Instructions may be provided in printed form or in the form of an electronic medium such as a floppy disc, CD, or DVD, or in the form of a website address where such instructions may be obtained. Often, a unit dose of dalbavancin includes a dosage such that when administered to an individual, a therapeutically or prophylactically effective plasma level of dalbavancin is maintained in the individual for at least 5 days. In some embodiments, a kit includes two unit dosages to be administered at least 5 days apart, often about one week apart, often including a first dosage of dalbavancin that is about 1.5 to about 3 times higher than the second dosage. Dalbavancin is often included as a sterile aqueous pharmaceutical composition or dry powder (*e.g.*, lyophilized) composition.

[0099] Suitable packaging is provided. As used herein, “packaging” refers to a solid matrix or material customarily used in a system and capable of holding within fixed limits a dalbavancin composition suitable for administration to an individual. Such materials include glass and plastic (*e.g.*, polyethylene, polypropylene, and polycarbonate) bottles, vials, paper, plastic, and plastic-foil laminated envelopes and the like. If e-beam sterilization techniques are employed, the packaging should have sufficiently low density to permit sterilization of the contents.

[0100] Kits may also optionally include equipment for administration of dalbavancin, such as, for example, syringes or equipment for intravenous administration, and/or a sterile solution, *e.g.*, a diluent such as 5% dextrose, for preparing a dry powder (*e.g.*, lyophilized) composition for administration.

[0101] Kits of the invention may include, in addition to dalbavancin, a non-dalbavancin antibiotic or mixture of non-dalbavancin antibiotics, for use with dalbavancin as described in the methods above.

[0102] In the examples below, the following abbreviations have the following meanings. If an abbreviation is not defined, it has its generally accepted meaning.

AcOH	=	acetic acid
AcONa	=	sodium acetate
aq.	=	aqueous
AST	=	aspartate amino transferase
ALT	=	alanine amino transferase
BV	=	bed volume
CV	=	coefficient of variation
d	=	diameter
D	=	dalton
DCC	=	dicyclohexylcarbodiimide
DMEPA	=	3-(dimethylamino)-propylamine
DMSO	=	dimethyl sulfonamide
eq	=	equivalents
EU	=	endotoxin units
g	=	gram
GC	=	gas chromatography
HCl	=	hydrochloric acid
H ₂ O	=	water
HOBT	=	1-hydroxybenzothiazole hydrate
HPLC	=	high performance liquid chromatography
H ₂ SO ₄	=	sulfuric acid
IPA	=	isopropylamine
IU	=	international unit
KF	=	potassium fluoride
Kg	=	kilogram
L	=	liter
LC/MS/MS	=	liquid chromatography/mass spec/mass spec
LDH	=	lactate dehydrogenase
LSC	=	liquid scintillation counting
m ³	=	cubic meter
MeOH	=	methanol
mg	=	milligram
mL	=	milliliter
mol	=	molar
MW	=	molecular weight

N	=	normal
NaOH	=	sodium hydroxide
NMP	=	N-methyl-2-pyrrolidone
QTD	=	quantitative tissue distribution
Rt	=	retention time
sd	=	standard deviation
TEA	=	triethylamine

[0103] The following examples are intended to illustrate but not limit the invention.

EXAMPLES

Example 1. Efficacy and Safety of Once Weekly Dalbavancin In Deep Skin and Soft Tissue Infections

[0104] This randomized, controlled study evaluated the safety and efficacy of two dose regimens of dalbavancin. Adult patients with skin and soft tissue infections (SSTI) involving deep skin structures or requiring surgical intervention were randomized to three groups: Study arm 1 received 1100 mg of dalbavancin via intravenous injection (IV) on day 1; Study arm 2 received 1g of dalbavancin IV on day 1 and 500 mg of dalbavancin IV on day 8; Study arm 3 received “standard of care.” Clinical and microbiological response and adverse events were assessed.

Populations for analysis

[0105] There were 62 patients randomized into the study; all received at least one dose of study medication. Four study populations were evaluated for safety and efficacy and were defined as follows: The intent-to-treat (ITT) population included all patients who received at least one dose of study drug (all randomized study

subjects). The microbiological intent-to-treat (MITT) population were all ITT patients who had a culture-confirmed Gram-positive pathogen at baseline. The clinically-evaluable population were defined as those who 1) fulfilled all study entry criteria, 2) had no change in antimicrobial therapy for Gram-positive infection following Day 4, except for oral step-down therapy (only applied to standard of care group), 3) returned for the follow-up (FU) assessment visit (unless a treatment failure), and 4) did not receive a non-protocol approved concomitant antimicrobial (unless a treatment failure). The microbiologically-evaluable population was the subset of clinically-evaluable patients who had a culture-confirmed Gram-positive pathogen at baseline.

[0106] The study populations are shown in **Table 2**.

Table 2. Study Populations for Dalbavancin SSTI Treatment

Populations	Study arm 1 Dalbavancin 1100mg day 1	Study arm 2 Dalbavancin 1000mg day 1, 500mg day 8	Study arm 3 “Standard of care”
Randomized ITT	20	21	21
Treated	20 (100%)	21 (100%)	21 (100%)
Completed Study	18/20 (90%)	20/21 (95.2%)	21/21 (100%)
Clinically eval at EOT	16/20 (80%)	17/21 (81%)	21/21 (100%)
Clinically eval at FU	13/20 (65%)	17/21 (81%)	21/21 (100%)
MITT	14/20 (70%)	13/21 (61.9%)	14/21 (66.7%)
Micro eval at EOT	13/20 (65%)	11/21 (52.4%)	14/21 (66.7%)
Micro eval at FU	11/20 (55%)	11/21 (52.4%)	14/21 (66.7%)

ITT - intent-to-treat

MITT - subset of ITT population with culture confirmed Gram-positive infection

EOT - end of treatment

FU - follow up

[0107] The median age of the subjects was 50-55 years (range 18-86 years). There were no apparent differences in age across the treatment arms. There were differences in gender across treatment arms, but overall the study enrolled equal numbers of men and women. The patient population was predominantly Caucasian. These results were consistent for both the ITT and clinically evaluable populations.

[0108] 62 patients were enrolled, 20 in Study arm 1 and 21 each in Study arms 2 and 3. The most common comparators for standard of care were clindamycin, ceftriaxone, vancomycin and cefazolin. Mean duration of treatment in Study arm 3 was 15 days.

Baseline pathogens and susceptibility

[0109] Of the 62 ITT patients, 66% (14 single-dose dalbavancin, 13 two-dose dalbavancin, 14 standard of care) had a pretherapy Gram-positive pathogen isolated (MITT population). The most common pathogen was *S. aureus*. The distribution of pathogens at baseline is shown in Table 3.

Table 3. Baseline Gram-positive Pathogens and Dalbavancin MIC Range for the MITT Population

Number with Pathogen (MIC)	Single dose Dalbavancin (1100 mg) (N=14)	Two-dose Dalbavancin (1000/500 mg) (N=13)	Standard of Care Regimens (N=14)
All <i>S. aureus</i>	13 (0.12)	11 (0.12)	10 (0.016-0.25)
Methicillin-sensitive	7	6	8
Methicillin-resistant	6	5	2
Group B streptococcus	0	2 (0.016)	2 (0.016)
<i>Streptococcus pyogenes</i>	0	1 (0.016)	1 (0.016)
Miscellaneous <i>Streptococcus</i> and	3 (0.016)	2 (0.016)	4 (0.016)

nontypeable strains			
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Clinical and microbiological responses

[0110] The effectiveness of the three treatment regimens was determined by assessing the patients' clinical response and the documented or presumed microbiological responses. The primary efficacy endpoint was clinical response at the follow-up visit for the clinically evaluable population. Clinical response, for both EOT and FU visits, was categorized as success (cure or improvement) or failure (including indeterminate results). Patients classified as successes must not have received additional systemic antibacterial treatment for their infection. Failure was defined as persistence of one or more local or systemic signs and symptoms of SSTI such that treatment with new or additional systemic antibacterial agents was required for the SSTI.

[0111] Microbiological outcome, a secondary efficacy variable, was assessed in the subpopulation of patients who had microbiologically documented SSTI (*i.e.*, at least one identified baseline pathogen). A microbiologic response was assessed for each Gram-positive pathogen identified at baseline (*i.e.*, eradication, presumed eradication, persistence, presumed persistence). For patients for whom follow-up cultures were not performed, the microbiologic responses for baseline pathogens were presumed on the basis of the clinical response. Microbiologic response by patient at the EOT and FU visits was graded as success (*i.e.*, all Gram-positive organisms eradicated or presumed eradicated) or failure (*i.e.*, at least one Gram-positive organism persisted or presumed to have persisted, multiple pathogens with partial eradication). At both the EOT and FU visits, colonization and superinfection were assessed. At the FU visit, a patient's bacteriological response could also include recurrence.

Clinical efficacy

[0112] Clinical success rates are shown in **Table 4**. In the clinically-evaluable population, 61.5% of patients in the single-dose dalbavancin, 94.1% in the two-dose dalbavancin, and 76.2% in the standard of care group were classified as successes at the time of the FU assessment. In an exploratory subanalysis of those patients categorized with deep or complicated SSTI at baseline, two-dose dalbavancin therapy also provided a higher clinical success rate (93.8%), compared with the single-dose dalbavancin and standard of care therapies, 58.3% and 73.7%, respectively.

[0113] Similar success rates at both the EOT and FU assessments were found in the supportive ITT and microbiologically-evaluable populations with a consistent trend towards a more favorable response following treatment with two-dose dalbavancin (**Table 3**). For the MITT population, clinical success rates at the FU assessment for those with methicillin-resistant *S. aureus* (MRSA) were 50% (3/6) for single-dose dalbavancin, 80% (4/5) for two-dose dalbavancin, and 50% (1/2) for patients treated with a standard of care regimen

Table 4. Clinical Success Rates by Analysis Population and Treatment Group

Population	Single-dose (1100 mg) Dalbavancin	Two-dose (1000/500 mg) Dalbavancin	Standard of Care Regimens
ITT at EOT	15/20 (75.0)	19/21 (90.5)	17/21 (81.0)
ITT at FU	12/20 (60.0)	19/21 (90.5)	16/21 (76.2)
MITT at EOT	10/14 (71.4)	12/13 (92.3)	10/14 (71.4)
MITT at FU	7/14 (50.0)	12/13 (92.3)	9/14 (64.3)
Clinically evaluable at EOT	13/16 (61.5)	16/17 (94.1)	17/21 (81.0)
Clinically evaluable at FU	8/13 (61.5)	16/17 (94.1)	16/21 (76.2)
Microbiologically evaluable at EOT	10/13 (76.9)	10/11 (90.9)	10/14 (71.4)
Microbiologically evaluable at FU	6/11 (54.5)	10/11 (90.9)	9/14 (64.3)

Microbiologic efficacy

[0114] The success rates of the different treatment regimes with respect to different pathogens is shown in **Table 5**. For the microbiologically-evaluable population, eradication/presumed eradication rates at the FU assessment for all

organisms were 58.3% (7/12) for single-dose dalbavancin, 92.3% (12/13) for two-dose dalbavancin, and 70.6% (12/17) for patients in the standard of care group. For isolates that persisted, there was no change in dalbavancin MIC. At FU, *S. aureus* eradication rates were higher for the two-dose dalbavancin group (90%) compared with single-dose dalbavancin (50%) and standard of care (60%) treatments. Similar findings were observed for the MITT population; two-dose dalbavancin eradicated 80% of MRSA isolates (**Table 5**).

Table 5. Success Rates by Pathogen for Microbiologically ITT Population at FU Assessment

	Single-dose (1100 mg) Dalbavancin	Two-dose (1000/500 mg) Dalbavancin	Standard of Care Regimens
Total organisms	7/16 (43.8%)	14/16 (87.5%)	12/17 (70.6%)
All <i>S. aureus</i>	5/13 (38%)	9/11 (82%)	6/10 (60%)
Methicillin-sensitive	2/7 (29%)	5/6 (83%)	5/8 (63%)
Methicillin-resistant	3/6 (50%)	4/5 (80%)	1/2 (50%)
Miscellaneous streptococcus species	2/3 (67%)	4/4 (100%)	5/7 (71%)

[0115] For the microbiologically-evaluable and MITT populations, the microbiological success rates at EOT and FU are summarized in **Table 6**. Comparable microbiologic success rates were reported at both visits for patients treated with two-dose dalbavancin and standard of care regimens (approximately 64% to 77%), whereas those given a single dose of dalbavancin had lower rates of success (<40%). The microbiologic success rates at EOT/FU in the microbiologically-evaluable population paralleled clinical response findings: 38.5%/27.3% for single-dose dalbavancin, 72.7%/72.7% for two-dose dalbavancin, and 71.4%/64.3% for standard of care therapy. Similar findings were observed for the MITT population (data not shown).

Table 6. Microbiologic Success Rates

	Single-dose (1100 mg) Dalbavancin	Two-dose (1000/500 mg) Dalbavancin	Standard of Care Regimens
MITT population			
EOT	5/14 (35.7)	10/13 (76.9)	10/14 (71.4)
FU	3/14 (21.4)	9/13 (69.2)	9/14 (64.3)
Microbiologically evaluable population			
EOT	5/13 (38.5)	8/11 (72.7)	10/14 (71.4)
FU	3/11 (27.3)	8/11 (72.7)	9/14 (64.3)

Pharmacokinetic analysis

[0116] For patients randomized to the dalbavancin treatment groups, 5 ml of blood was obtained on Day 8 for determination of dalbavancin plasma concentrations. For patients randomized to receive a 500 mg dalbavancin dose on Day 8, blood was obtained just prior to administration of the second dose. Additional 5 ml blood samples were obtained on Days 10 and 24 for patients were randomized to the single-dose dalbavancin group and on Days 20 and 34 for those who received two doses of dalbavancin.

[0117] Dalbavancin plasma concentrations were determined using validated liquid chromatography and mass spectrophotometer methods. The lower limit of quantitation was 500 ng/ml for plasma.

[0118] Mean dalbavancin concentrations collected on Study days 8, 10, and 24 in the single-dose regimen were 31.1 ± 7.1 , 25.2 ± 4.8 , and 10.2 ± 3.5 mg/l (mean \pm SD), respectively. Dalbavancin concentrations following the two-dose regimen on Study days 8 (prior to the second dose), 20, and 34 were 30.4 ± 8.2 , 21.2 ± 10.0 , and 9.0 ± 4.4 mg/l, respectively. As expected, all patients had dalbavancin concentrations of greater than 20 mg/l through the first week following the first dose, and levels above 20 mg/l were maintained for an additional week with an additional dose of 500 mg IV on day 8. Generally, minimum bactericidal concentrations are about 4 to 10 mg/l.

Safety evaluation

[0119] Each patient who received at least one dose of study drug (ITT population) was evaluated for drug safety through monitoring of adverse events (AE), including abnormal clinical laboratory test results and vital signs. AE were rated by the investigator as to their severity (mild, moderate, severe, life-threatening), and by the relationship to the study drug (not related, unlikely related, possibly related, or probably related).

[0120] A summary of the AE data is presented in **Table 7**. The majority of adverse reactions (90%) were considered mild to moderate in severity. All serious adverse reactions (8 events in 5 patients) were unrelated to study drug treatment. Approximately 59% of all patients who reported at least one treatment-emergent AE (19 single-dose dalbavancin, 16 two-dose dalbavancin, 21 standard of care) experienced an event that was categorized by the investigator as possibly or probably related to study drug. Specifically, drug-related AEs were reported in 11 (55%) single-dose dalbavancin, 10 (48%) two-dalbavancin, and 12 (57%) standard of care patients. The most frequently reported drug-related AE in both the dalbavancin and standard of care treatment groups were diarrhea and nausea. A summary of types of AEs observed for the different treatment groups is presented in **Table 8**.

[0121] No dalbavancin-treated patient discontinued treatment prematurely due to an AE. Three of 21 (14%) patients on a standard of care regiment discontinued treatment prematurely due to an AE, including one patient who developed urticaria on Day 1 which was probably drug related and two patients who had AE unrelated to study drug (superinfection with *P. aeruginosa* and elevated vancomycin trough level).

Table 7. Summary of Adverse Event (AE) Data

	Single-dose dalbavancin (N=20)	Two-dose dalbavancin (N=21)	Standard of care (N=21)
≥1 AE	95%	76.2%	100%
%AE severe	15%	9.5%	4.8%
≥1 AE possibly/probably related to treatment	55%	48%	57%
AE leading to discontinuation of study medication	0	0	14.3%
≥1 severe AE	2 (10%)	2 (9.5%)	1 (4.8%)

Table 8. Most Common Adverse Events

	Single-dose dalbavancin (N=20)	Two-dose dalbavancin (N=21)	Standard of care (N=21)
Diarrhea	20%	9.5%	28.6%
Nausea	10%	28.6%	9.5%
Hyperglycemia	5%	14.3%	19%
Limb Pain	10%	9.5%	9.5%
Vomiting	10%	14.3%	4.8%
Constipation	5%	4.8%	14.3%

Discussion

[0122] This open-label, randomized Phase II trial shows that dalbavancin is effective for the treatment of adults with SSTI. The majority of enrolled patients had deep or complicated infections (>90%) and infections that required surgical intervention (~70%), while approximately 45% had underlying diabetes mellitus.

[0123] Two weekly doses of dalbavancin had a numerically higher clinical response rate than either a single-dose of dalbavancin or the standard of care regimen. Data from both ITT and clinically-evaluable populations suggests that a regimen of two sequential weekly injectable doses of dalbavancin (1000 mg, 500 mg weekly) is effective in the treatment of SSTIs. The standard of care group was treated for a median duration of 13 days. At follow-up, 94% of clinically-evaluable patients treated with two-dose dalbavancin were considered clinical successes versus 76% of those given a standard of care regimen and 61.5% of patients receiving single-dose dalbavancin.

[0124] *S. aureus* was the most frequently isolated organism at baseline. In this trial, approximately 83% of patients were infected with *S. aureus* and 38% of all *S. aureus* strains were MRSA. Most infections (80%) were caused by a single pathogen. The MICs for dalbavancin against Gram-positive isolates, including MRSA, ranged from 0.016 to 0.25 mg/L.

[0125] Microbiological success rates paralleled those of clinical response for the clinically-evaluable population. For all organisms combined, treatment with the two-dose weekly dalbavancin regimen provided higher eradication rates at the 2 week post-therapy assessment (92%) compared with single-dose dalbavancin (58%) and standard of care therapies (71%). Overall, rates of *S. aureus* eradication were observed in 90%, 50%, and 60% of patients, respectively. For the MITT population, rates of eradication for MRSA were 80% for the two-dose dalbavancin regimen versus 50% for both the single-dose dalbavancin and standard of care therapies.

[0126] Concentrations of dalbavancin obtained at the end of the single-dose and two-dose weekly treatment periods (Day 10 or Day 20, respectively) were similar suggesting little drug accumulation following the second weekly dose. The higher rate of clinical success observed with the two-dose regimen is suggestive of time-dependent killing wherein sustained levels of drug or drug exposure were provided with two doses of dalbavancin separated by one week. Dalbavancin plasma levels measured at the end of the weekly dosing interval were substantially greater than the

reported MIC₉₀ for pathogens responsible for the majority of SSTIs (<0.03 to 0.5 mg/L), including those found in this trial. These levels were also above the minimum bactericidal concentrations of 4 to 10 mg/l.

[0127] The overall rate of adverse reactions was similar for both dalbavancin regimens and the standard of care group. Gastrointestinal drug-related adverse events (*i.e.*, diarrhea and nausea) were most commonly reported across the three treatment groups. The majority of these events were mild and self-limited. No dalbavancin-treated patient was withdrawn from the study early due to an adverse reaction, nor were any serious adverse events attributable to the glycopeptide reported, yet 14% of the standard of care group withdrew due to adverse effects. The novel dosage regimen thus had reduced adverse side effects in comparison to the standard of care. The data from this trial found no evidence that dalbavancin induces any degree of clinically significant hepatotoxicity or nephrotoxicity.

[0128] The two-dose dalbavancin regimen appears effective for treatment of patients with complicated SSTIs. Dalbavancin at both doses was well tolerated in this clinical trial, with an adverse event profile similar to that of the standard of care group.

Example 2. Pharmacokinetics and Renal Excretion of Dalbavancin in Healthy Subjects

[0129] The primary objectives of this study were to characterize the pharmacokinetics of dalbavancin and to calculate the extent of renal excretion in healthy subjects receiving a therapeutic dose of the drug. This was an open label, non-comparative, study.

Study drug treatment

[0130] Healthy male or female subjects between 18 and 65 years of age were administered a single 1000 mg IV dose of dalbavancin infused over 30 minutes.

[0131] Six subjects, one female and five male, were enrolled, received study medication, and completed all aspects of the study. Three subjects were Caucasian and three subjects were African-American. Mean age was 29.8 years (range 22 to 63). Mean height was 68.6 inches (range 63 to 75) and mean weight was 179.6 lbs (140 to 244).

Pharmacokinetics

[0132] Blood and urine (24-hr collections) were collected on study days 1, 2, 3, 4, 5, 6, 7, 14, 21, 28, and 42. Blood samples were drawn into heparinized tubes and centrifuged. Plasma was separated and stored frozen at -20°C until time of assay. Plasma and urine samples were assayed for dalbavancin using validated LC/MS/MS methods. The lower limit of quantitation of the assay was 500 ng/mL for urine and plasma.

[0133] Dalbavancin pharmacokinetic parameters were estimated by non-compartmental methods using the WinNonlin™ software (Pharsight Corporation). The peak concentration (C_{max}) values were obtained directly from the observed data. The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule. Clearance (CL) was computed as dose/AUC. The elimination half life ($t_{1/2}$) was estimated by linear regression of the log-linear portion of the log concentration versus time curve. Estimates of the volume of distribution (V_z) was calculated using the regression parameters, while the volume of distribution at steady state (V_{ss}) was calculated from the area under the first moment curve (AUMC) multiplied by the dose and divided by AUC. The cumulative amount of dalbavancin excreted in urine was determined as the integrand of the urine excretion rate (AURC). CL_R , or renal clearance, was calculated as the ratio: $CL_R = AURC/AUC$.

[0134] Plasma concentrations of dalbavancin versus time are shown for all subjects in **Figure 1**. Pharmacokinetic parameters are presented in **Table 9**. Concentrations were similar across all subjects. Peak plasma concentrations were approximately 300 mg/L and were achieved immediately following the end of infusion. Dalbavancin shows an apparent volume of distribution of more than 10 L and is assumed to be well distributed in the extracellular fluid.

[0135] Dalbavancin was slowly eliminated with a $t_{1/2}$ of 9-12 days. The total drug clearance was 0.0431 ± 0.0074 L/hr. The estimated fraction of drug excreted unchanged into urine was 42% of the administered dose, and renal clearance was estimated as 0.018 L/h. The variability observed across subjects was low with a coefficient of variation of less than 22% across all pharmacokinetic parameters.

Table 9. Pharmacokinetic parameters

	C_{max}	t_{1/2}	AUC	V_z	CL	V_{ss}	AURC	% Renal	CL_R
	mg/L	h	mg.h/L	L	L/h	L	mg	Excretion	L/h
Mean	301	257	23843	16.0	0.0431	11.5	419	41.9	0.0181
Sd	65	21	4526	3.1	0.0074	2.13	27	2.7	0.0036
CV%	21.6	8.1	19.0	19.5	17.1	18.6	6.4	6.4	20.1
Min	243	227	19844	11.7	0.0332	8.58	379	37.9	0.0130
Max	394	282	30100	19.6	0.0504	13.9	448	44.8	0.0226

Safety assessments

[0136] Adverse events were recorded and assessed for severity and relationship to study drug. Laboratory data (chemistry panel, CBC with differential, urinalysis) were collected and assessed for changes from baseline and out-of-range values. ECG, physical examination, and vital signs were obtained, and changes from baseline were assessed.

[0137] Dalbavancin was well-tolerated in this study. No subject deaths or serious adverse events were reported during this study and no subject was prematurely withdrawn from study due to an AE.

[0138] All volunteers reported at least one AE, all of mild intensity. Three volunteers reported AEs that were possibly related to study medication: elevated ALT (value 46 IU/L, upper limit of normal 40 IU/L) in one subject; eosinophilia (value $0.5 \times 10^3/\mu\text{L}$, upper limit of normal $0.4 \times 10^3/\mu\text{L}$), elevated LDH (value 303 IU/L, upper limit of normal 90 IU/L), elevated ALT (value 54 IU/L, upper limit of normally 40 IU/L), elevated AST (value 42 IU/L, upper limit of normal 40 IU/L) all in one subject; and tinnitus in one subject.

[0139] No trends were seen for post-baseline hematology, chemistry, vital signs, and ECG results.

Discussion

[0140] A single 1000 mg IV dose of dalbavancin was well-tolerated. Following a single intravenous infusion of 1000 mg, plasma concentrations of dalbavancin above 45 mg/l are maintained for at least seven days. This is above concentrations known to be bactericidal (4-32 mg/l). This supports the use of dalbavancin as a once-weekly regimen. The urinary elimination profile indicates that renal excretion is an important elimination pathway, with approximately 40% excreted in urine. This finding is consistent with observations in animals. Since the kidneys are not the exclusive elimination route, a dosing adjustment for dalbavancin may not be necessary in renally impaired patients.

Example 3. Protein Binding of Dalbavancin using Isothermal Titration Microcalorimetry

[0141] Binding of dalbavancin to proteins was measured by isothermal titration microcalorimetry (ITC) in 20 mM phosphate, 150 mM NaCl, pH 7.4 at 25 and 37 °C using a Microcal VP-ITC instrument. In a typical experiment, 25 x 10 μl of protein (~150 μM) was injected into a calorimeter cell containing dalbavancin solution (~5

μM). Actual protein and dalbavancin concentrations were determined by measuring absorbance at 280 nm. Control experiments included injections of protein into buffer (in the absence of dalbavancin) to account for the heats of dilution of protein under identical conditions. For comparison, similar experiments with some necessary modifications were performed using teicoplanin.

[0142] Experiments with dalbavancin were conducted with each of the following proteins: human albumin; dog albumin; rat albumin; bovine albumin; and human α-glycoprotein. Teicoplanin was studied with human albumin and α-glycoprotein. A comparison of binding affinities at two different temperatures is shown in Table 10.

Table 10. Comparison of apparent binding affinities (K_a , $\times 10^5 \text{ M}^{-1}$)

	25 °C	37 °C
<u>Dalbavancin</u>		
Human albumin	1.35 (± 0.2)	1.33 (± 0.15)
Rat albumin	3.1 (± 0.5)	2.8 (± 1.8)
Dog albumin	0.62 (± 0.09)	0.50 (± 0.13)
Bovine albumin	1.38 (± 0.14)	
α-glycoprotein	1.84 (± 0.36)	4.8 (± 2.3)
<u>Teicoplanin</u>		
Human albumin	0.96 (± 0.08)	
α-glycoprotein	0.07 (± 0.01)	

The ± errors quoted are the standard deviations obtained from the fitting routine.

[0143] Integrated heat effects, after correction for heats of dilution, were analyzed by non-linear regression using a simple single-site binding model with the standard Microcal ORIGIN software package. Raw data (μcal/sec) for each injection were integrated to give the total heat effect per addition, then divided by amount of injectant to give kcal/mole of injectant. The same integration was applied for control dilution effects, and this was subtracted from the actual titration data. This provided a differential of the binding curve in which the extent of binding is proportional to the total heat liberated (or absorbed). This was then analyzed by non-linear regression

methods in terms of various standard binding models. The simplest model assumes simple non-competitive binding equilibrium, and gives three parameters:

$K_a (=1/K_{diss})$ is the binding association (dissociation constant)

ΔH = the enthalpy of binding (the size of signal related to binding)

N = number of binding sites (assuming the binding model is correct)

[0144] Assuming non-competitive binding, N is the (relative) number of moles of injectant required to saturate all the available binding sites in the sample. For the dalbavancin experiments, dalbavancin is the "sample" and the protein (HSA, etc.) is the "injectant." These preliminary results indicate that the binding is relatively weak and, because of the poor solubility of dalbavancin, it is difficult to determine the binding stoichiometry (N) unambiguously. However, as seen in **Figure 2**, in all cases, the data fits well with $N < 1$ (i.e., less than one to one protein to dalbavancin). Consequently, a value of $N = 0.5$ means that it only takes half as many moles of protein to bind all the dalbavancin than would be expected. In other words, each protein apparently binds two dalbavancin molecules. It is possible that dalbavancin forms a dimer that binds 1:1 with a protein. Results of binding stoichiometry modeling suggests that two dalbavancin molecules are bound to one molecule of protein, unlike teicoplanin, which exhibits 1:1 binding.

[0145] **Table 11** presents the calculated percent bound for antibiotic concentrations in the range 1-500 μM , assuming physiological concentrations of human serum albumin ($6 \times 10^{-4} \text{ M}$) and α -glycopeptide ($1.5 \times 10^{-5} \text{ M}$). To relate this to the clinical situation, the peak concentration of dalbavancin in man is approximately 300 mg/L, or 165 μM .

Table 11. Calculated percent Binding of Teicoplanin and Dalbavancin to Plasma Proteins

Concentration of antibiotic (μ M)	Dalbavancin	Teicoplanin
Human albumin		
1	98.8	98.3
10	98.8	98.3
100	98.7	98.0
165	98.6	ND
250	98.5	ND
500	98.0	ND
Human α -glycoprotein		
1	73.0	9.6
10	68.2	9.1
100	26.2	6.0
165	16.9	ND
250	11.4	ND
500	5.9	ND

ND= Not done

[0146] In these experiments, the binding of dalbavancin to human serum albumin exceeds 98%. The fraction bound is fairly constant across the selected range of dalbavancin concentrations, i.e. 1-500 μ M. This range encompasses the therapeutic concentrations in man. Binding of dalbavancin to α -glycoprotein is much greater than that of teicoplanin. Dalbavancin demonstrates high capacity and low affinity for plasma proteins of different origin, with similar K_a values across proteins from all species tested. These results help to explain some of the unique pharmacokinetic characteristics of dalbavancin. The binding and formation of a 2:1 dalbavancin:protein complex also explains the prolonged half-life, and the apparent volume of distribution, which approximates extracellular water volume. The low affinity helps explain the observed *in vivo* activity, which greatly exceeds what would be expected for a compound with a free fraction close to 1%. The high capacity for plasma proteins helps to explain the relatively high plasma concentrations achieved in spite of poor solubility of the compound at physiological pH.

Example 4. Pharmacokinetic Attributes and Tissue Distribution of Dalbavancin in Rats

[0147] Two studies were performed in rats administered a single IV infusion of 20 mg/kg [³H]-dalbavancin. Excreta and more than 40 different tissues were collected through 70 days post-administration, and the tissue distribution and pharmacokinetics of drug-derived radioactivity were determined.

[0148] HPLC-purified [³H]-dalbavancin was used for these studies. Radiolabeled drug was produced via tritium exchange and purified by HPLC.

Rat mass balance study

[0149] Mass balance studies were conducted to determine the excretion pattern of dalbavancin following a single intravenous (IV) infusion of dalbavancin in male rats.

[0150] Fifteen male Sprague-Dawley rats received a single IV dose of ³H-dalbavancin (20 mg/kg, 100 μ Ci/rat). Following dose administration, urine and feces were collected at 24 hour intervals to 14, 36, and 70 days after the dose (3 rats/final collection time). Water and methanol cage washes were also collected. Carcasses were analyzed at the end of the collection period. All samples were analyzed for total radioactivity content by liquid scintillation counting (LSC).

[0151] Following IV administration of ³H-dalbavancin in rats, drug-derived radioactivity was eliminated in both urine (~ 2/3 of excreted radioactivity) and feces (~ 1/3 of excreted radioactivity). Approximately half of the radioactivity administered was eliminated in the urine and feces within the first week, which is consistent with a plasma t_{1/2} of approximately 1 week. At 70 days post dose, only 4.5% of the dose remained in the carcass. Negligible radioactivity was recovered in the cage washes. Virtually all of the administered radioactivity was accounted for (urine, feces, carcass, cage washes, and tritium exchange) during the study.

Rat quantitative tissue distribution (QTD) study

[0152] Quantitative tissue distribution studies were conducted to assess the tissue distribution of dalbavancin following a single IV infusion of dalbavancin to male rats.

[0153] Forty-one male Sprague-Dawley rats received a single IV infusion of ^3H -dalbavancin (20 mg/kg, 50 $\mu\text{Ci/rat}$). Rats (3 per time-point) were euthanized at 12, 24, 48, 72, 96, 120, 144, 168, 336, 840, 1176 and 1680 hours post dose for collection of blood, plasma, and tissues (including carcass). All samples were analyzed by LSC.

[0154] Concentration-time profiles were determined for more than 40 tissues, including kidney, liver, spleen, blood, plasma, lung, and skin. Concentrations and $t_{1/2}$ values of drug-derived radioactivity in tissues, including skin, were comparable to those observed in plasma. Dalbavancin was found to be rapidly and extensively distributed with all tissues having quantifiable concentrations of drug-derived radioactivity within 12 hours after post-infusion. Most tissues reached maximum concentration (C_{max}) within 24 h after the dose. Recovered radioactivity after 5 days was <5% of the dose in any single tissue. By 70 days after the dose, only the carcass retained > 1% (2.34%) of the administered radioactivity. Thus, dalbavancin did not accumulate in any single tissue, organ, or blood cellular component. Concentrations of radioactivity in the CNS were low but detectable in this healthy animal model. Dalbavancin was found to penetrate the skin with concentrations of drug-derived radioactivity that were as high as or higher than in plasma. Blood to plasma ratio of drug-derived radioactivity remained relatively constant over time and was <1.

[0155] As part of the QTD studies, bile samples were collected from bile duct cannulated rats (4 animals) through 384 h (16 days) post-dose. Almost 11% of the dose was recovered in the bile over 384 h after the dose. This represents the majority of the drug-derived radioactivity found in feces.

Example 5. Quantitative determination of dalbavancin in plasma by HPLC-MS/MS

[0156] A HPLC-MS/MS method was developed for quantitative measurement of dalbavancin in plasma, as described below.

Preparation of dalbavancin calibration and quality control standards

[0157] Stock solutions of dalbavancin were prepared by dissolving dalbavancin in deionized water to prepare a 1000 µg/ml solution, followed by serial dilutions in deionized water to prepare 500, 50 and 10 µg/ml solutions.

[0158] Calibration standards of 100, 60, and 40 µg/ml dalbavancin concentration were prepared by spiking human plasma with appropriate volumes of a 1000 µg/ml dalbavancin stock solution prepared as described above. Calibration standards of 20 and 10 µg/ml concentration were prepared by spiking human plasma with appropriate volumes of a 500 µg/ml dalbavancin stock solution, and a calibration standard of 0.5 µg/ml was prepared by spiking human plasma with an appropriate volume of a 10 µg/ml stock solution.

[0159] Quality control standards of 90 and 30 µg/ml dalbavancin were prepared by spiking human plasma with an appropriate volume of a 1000 µg/ml dalbavancin stock solution prepared as described above. A quality control standard of 1.5 µg/ml was prepared by spiking human plasma with an appropriate volume of a 50 µg/ml solution.

Preparation of internal standard working solution

[0160] A 30 µg/ml working solution of internal standard BI-K0098, which is the diethyl-amino-propyl-amino derivative of A-40926, was prepared as follows. Approximately 10mg of BI-K0098 was dissolved in approximately 10 ml of mobile phase A (80% of 10mM Ammonium Formiate/Formic Acid, pH 3 (v/v), 10% of

Acetonitrile (v/v), and 10% 2-Propanol (v/v)) to make a 1000 $\mu\text{g/ml}$ internal standard stock solution. The stock solution (300 μl) was then diluted to a volume of 10 ml with mobile phase A to make a 30 $\mu\text{g/ml}$ internal standard solution.

Preparation of samples for analysis

[0161] Samples were prepared as follows for quantitative determination of dalbavancin concentration in plasma. To 50 μl of calibration or quality control standards prepared as described above, 100 μl of internal standard working standard solution was added and mixed. The mixture was permitted to equilibrate for five minutes at room temperature, followed by addition of 250 μl of acetonitrile. The mixture was then vortexed for 10 seconds, followed by centrifugation for 1 minute at about 10,000 rpm on an ALC micro-centrifuge 4214. Supernatants were transferred to clean tubes and evaporated to dryness in a Savant Speed-Vac System at about 40 °C. Samples were then resuspended in 150 μl of mobile phase A.

Analytical method

[0162] 50 μl samples prepared for analysis as described above were injected into a Phenomenex Jupiter C₁₈ column (50 x 2 mm, C₁₈ 5 μm 300 A), and analyzed under gradient HPLC conditions at a flow rate of 0.3 ml/min. The gradient conditions were: initial, 80% mobile phase A/20% mobile phase B (20% 10mM Ammonium Formate/Formic Acid, pH 3 (v/v), 40% Acetonitrile (v/v), 40% 2-propanol (v/v)); 1 minute, 20% mobile phase A/80% mobile phase B; 2 minutes, 20% mobile phase A/80% mobile phase B; 2.5 minutes, back to initial conditions.

[0163] The HPLC system was coupled to a PE SCIEX API-2000 triple quadrupole mass spectrometer, with turbo ion spray operating in a positive ionization mode. Air was used to generate a spray in the ion source. Probe temperature was set at 500° C with nitrogen as curtain gas. Multiple reactions monitoring (MRM) was employed using nitrogen as collision gas. The analytes were detected by monitoring the following ion transitions: 909.3 Da \rightarrow 1429.3 Da for dalbavancin, and 923.3 Da \rightarrow

1457.3 Da for the internal standard (BI-K0098). To avoid mass spectrometer contamination, a post-column flow diversion in the first minute and 2.5 minutes after the beginning of the chromatographic run was performed.

[0164] Software Sample Control 1.4 was used for the acquisition of data analysis and software MacQuan 1.6 was used for the integration of chromatographic peaks and statistical data evaluation.

Calibration curves

[0165] Linearity of the assay method was assessed by assaying calibration standards to generate a calibration curve. The concentration of dalbavancin in a plasma sample was determined by calculating the peak area ratio between dalbavancin and the internal standard.

[0166] Calibration curves for dalbavancin concentrations over an analytical range of 0.5-100 μg dalbavancin/ml of human plasma were constructed using the equation $y = A + Bx$ (weighted $1/x$), where A represents intercept of the curve, B represents the slope of the curve, x represents the dalbavancin concentration of calibration standard ($\mu\text{g}/\text{ml}$), and y represents the peak area ratio of dalbanvancin to internal standard. Three separate calibration curves were constructed. The results showed that dalbavancin/internal standard area ratio and dalbavancin concentrations varied linearly over the analytical range. The lower limit of quantitation (LLOQ) was 0.5 μg dalbavancin per ml of human plasma. The slopes for the calibration curves were reproducible and their correlation coefficients were greater than 0.9995.

Stability of dalbavancin in plasma

[0167] The stability of dalbavancin in plasma samples was tested by analyzing three replicates quality control standards of human plasma samples, prepared as described above, at two different concentrations, 1.5 and 90 $\mu\text{g}/\text{ml}$. Detectable dalbavancin concentration was stable after three cycles of freeze-thaw treatment. Dalbavancin concentration in processed samples was stable after 24 hours at room

temperature. No reduction in dalbavancin concentration with respect to time zero samples was observed.

Example 6. Dalbavancin Mass Spectroscopy Analysis.

[0168] The nature of dalbavancin multimers in solution was investigated and the conditions influencing the population ratio of dalbavancin multimer to dalbavancin monomer were determined by electrospray ion mass spectroscopy (ESI-MS).

[0169] Experiments were performed using an Applied Biosystem API III+ mass spectrometer equipped with a TurboIonSpray source, a Triple Quadrupole analyzer, operating in positive ion mode. The optimized conditions are reported in **Table 12** below.

Table 12: Instrumental Conditions for Dalbavancin Analysis on Applied Biosystem API III+ Mass Spectrometer.

IonSpray source:		MS analyzer:	
IonSpray voltage	5000 V	Interface plate voltage	650 V
Orifice plate voltage	80 V	Q0 road offset voltage	40 V
Curtain gas flow	0.6 L/min	Q1 park mass	1000
Nebulizer gas flow	1.2 L/min	Q1 resolution	120.8
Liquid flow	5 μ L/min	Q1 delta mass	0.2
Interface heater	60°C	Q1 road offset voltage	27 V
Scan conditions (Q1 scan):		Lens 7 voltage	-50 V
		Q2 road offset voltage	-50 V
Step	0.1 amu	Q3 park mass	1000
		Q3 resolution	110
Dwell time	1 msec	Q3 delta mass	0
		Q3 road offset voltage	-70 V
		Lens 9 voltage	-250 V
		Faraday plate voltage	-250 V
		Channel electron multiplier voltage	-3800 V

Dalbavancin in solution

[0170] Instrumental parameters were tuned on a dalbavancin solution containing 0.1 mg/ml of dalbavancin dissolved in a 8:2 water:isopropanol solution. A spectrum of dalbavancin in solution was acquired in the range of 500-2000 amu following direct injection of the solution. The resulting spectrum, as seen in **Figure 3**, indicates the presence of dalbavancin multimers. As a non-limiting example, one trace of the spectrum is attributable to a homomultimer of B_0 , which is present as a $(2nM + y(^+3))$ ion species, where n is a positive integer indicating the multiplicity of the homomultimer, *e.g.*, $n = 1$ when the multimer is a homodimer and $n = 2$ when the multimer is a homotetramer, M indicates the mass of the monomer, $y = n$ and $^+3$ indicates a plus three ion charge. For example, the homodimer of B_0 is provided when $n = 1$, $y = 1$, and $M = \text{mass of } B_0$. This homodimer species is assigned to a $(2M + ^+3)$ ion trace in the mass spectrum.

Influence of dalbavancin concentration on the population ratio of dalbavancin multimer to monomer

[0171] The influence of dalbavancin concentration on the population ratio of multimer to monomer was evaluated by mass spectroscopy using the conditions described above. The spectra were acquired by direct infusion of dalbavancin solutions at concentrations of 20, 40, 60, and 80 $\mu\text{g/mL}$. The intensities of the main peaks were reported as a function of dalbavancin concentration and the population ratios of dalbavancin multimer to monomer were determined, as shown in **Figure 4**.

[0172] The data indicate that the population ratio of dalbavancin multimer to dalbavancin monomer increases with increasing concentration. This may help to explain the high drug loading capacities that may be administered to an individual. The role of multimer as a depot of monomer may decrease the tendency of higher concentration samples to form precipitates and enhance the concentrations which may be administered to an individual. The presence of multimers may also allow rapid administration of a dose of dalbavancin to an individual.

[0173] A non-limiting example of a method of determining the population ratio of dalbavancin multimer to monomer is provided, for example, by determining the ratio between peak intensities of ions A and B as shown in **Figure 3**. Dividing the intensity of peak A by the intensity of peak B provides one measure of the population ratio of dalbavancin multimer to monomer.

Influence of pH on the population ratio of dalbavancin multimer to monomer

[0174] The influence of solution pH on the population ratio of dalbavancin multimer to monomer was evaluated at the instrumental conditions described above and at the following solution pH values: 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5. The population ratio of dalbavancin multimer to monomer was determined at each of the pH values and plotted against pH, as seen in **Figure 5**. It was determined that the population ratio of dalbavancin multimer to monomer increases with increasing pH.

[0175] While not to be limited to theory, it is believed that ionic groups, such as a carboxylate group on a first dalbavancin monomer, aid in the stabilization of dalbavancin multimers by forming ionic interactions with oppositely charged ions, such as tertiary nitrogen groups, on a second dalbavancin monomer. Such ionic interactions can be influenced by pH. It is believed that the increasing tendency of dalbavancin to be present as a multimer at higher pH is indication that ionic interactions are important in multimer stabilization. In particular, it is believed that dalbavancin multimers are destabilized at lower pH, presumably due to interruption of the ionic interactions contributing to multimer stability as certain functional groups, such as carboxylate groups, may be protonated at lower pH values.

Influence of solution ionic strength on the population ratio of dalbavancin multimer to monomer

[0176] The influence of solution ionic strength on the population ratio of dalbavancin multimer to monomer was determined by mass spectrometry. The mass spectra were obtained in electrospray positive mode on a Finnigan LCQ^{Deca} ion trap

instrument previously tuned and calibrated in electrospray mode using Ultramark 1621, caffeine and MRFA (L-metionyl-arginyl-phenilalanyl-arginine). All the mass spectra were recorded using the conditions listed in **Table 13**. The sample parameters that were investigated are listed in **Table 14**.

Table 13. MS Conditions

Sample Inlet Conditions:	
Capillary Temperature (°C):	200
Sheat Gas (N ₂ , arbitrary units):	40
Sample Inlet Voltage Settings:	
Polarity:	positive
Source Voltage (kV):	4.70
Capillary Voltage (V):	34
Tube Lens Offset (V):	-60
Full Scan conditions:	
Scan range (amu):	500-2000
Number of microscans:	3
Maximum ion time (ms):	200
Zoom Scan conditions:	
Scan range (amu):	1218-1228
Number of microscans:	5
Maximum ion time (ms):	50

Table 14. Sample Parameters

Sample	μg/mL	Solvent	pH
Dalbavancin	100	COO ⁻ NH ₄ ⁺ 5 mM	5
	100	COO ⁻ NH ₄ ⁺ 50 mM	5
	100	COO ⁻ NH ₄ ⁺ 100 mM	5

[0177] Sample water solutions were infused at 10 μL/min via a Harward syringe pump and the mass spectra were obtained as seen in **Figures 6-8**.

[0178] The obtained spectra indicate that the population ratio of dalbavancin multimer to monomer is influenced by ionic strength. An increase in buffer concentration was found to correspond to a decrease in multimer mass traces and hence a decrease in dalbavancin multimer to monomer population ratio.

[0179] As mentioned, it is believed that ionic interactions are important in dalbavancin multimer stability. The fact that increasing ionic strength was

correlated with decreasing intensity of multimer mass traces substantiates the role of ionic interactions in multimer stability. However, as multimer mass traces were present even at higher ionic strengths, another, second, interaction may be involved in multimer stabilization.

[0180] While not bound to any theory, it is believed that hydrophobic interactions are important in stabilizing the multimer species of dalbavancin. If the stabilization of these non-covalent dalbavancin multimers was solely due to ionic interactions, it would be expected that an increase in ionic strength would result in total loss of multimer mass species. That is, it would be expected that as the ionic strength of the solution increases, the ionic interactions stabilizing the multimer would be disrupted by the increased population of ions in solution, with which the monomers would more readily associate. Consequently, the solution ionic strength would drive the multimers to disassociate into monomer components and the resulting mass spectra would be free of any multimer mass traces. However, even at high solution ionic strength (such as 100 mM ammonium formate), the presence of dalbavancin multimers is detected in the mass spectrum. Accordingly, the multimers of dalbavancin are deemed to be stabilized, at least in part, by hydrophobic interactions.

Structurally similar compound

[0181] It is believed that the improved efficacy of Dalbavancin is due at least in part to its ability to form multimers. It is thought that this unique characteristic is not shared even by very structurally similar compounds. A compound with a chemical structure similar to Dalbavancin was investigated by mass spectroscopy analysis for its ability to form multimers. The mass spectra were obtained in electrospray positive mode on a Finnigan LCQ^{Deca} ion trap instrument previously tuned and calibrated in electrospray mode using Ultramark 1621, caffeine and MRFA (L-metionyl-arginyl-phenylalanyl-arginine). All the mass spectra were recorded using the conditions listed in **Table 15**. The sample parameters that were investigated are

listed in **Table 16**. Sample water solutions were infused at 10 $\mu\text{L}/\text{min}$ via a Harvard syringe pump and mass spectra were obtained as seen in **Figures 9 and 10**.

Table 15. Mass Spectra Conditions

<i>Sample Inlet Conditions:</i>	
Capillary Temperature ($^{\circ}\text{C}$):	200
Sheat Gas (N_2 , arbitrary units):	40
<i>Sample Inlet Voltage Settings:</i>	
Polarity:	positive
Source Voltage (kV):	4.70
Capillary Voltage (V):	34
Tube Lens Offset (V):	-60
<i>Full Scan conditions:</i>	
Scan range (amu):	500-2000
Number of microscans:	3
Maximum ion time (ms):	200
<i>Zoom Scan conditions:</i>	
Scan range (amu):	1218-1228
Number of microscans:	5
Maximum ion time (ms):	50

Table 16. Sample Parameters

Sample	$\mu\text{g}/\text{mL}$	Solvent	pH
Teicoplanin	50	H_2O	n.a.
	100	H_2O	n.a.

n.a. = not adjusted

[0182] A similar glycopeptide antibiotic (teicoplanin) does not show multimeric complexes in solution at various concentrations. This supports the indication that structurally similar compounds fail to form multimeric species in solution, and that this phenomenon may play an important role in the activity of the dalbavancin.

Example 7. Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry of protein-dalbavancin complexes

[0183] 10 ul HSA, 0.150 mM was mixed with 10 ul dalbavancin solution (from 0.075 mM, 0.15 mM, 0.3 mM and 1.5 mM) and incubated for 60 min at 37°C. The samples were prepared for analysis using the dried droplet technique. Spectra were obtained on a BRUKER FLEX III, tof mass spectrometer previously tuned and calibrated using standard bovine serum albumin, acquiring and averaging spectra generated by 200 laser shots. Matrix: 9 parts of DHB-9 (2,5-dihydroxy-benzoic acid) saturated in acetonitrile/H₂O (50:50), 1 part of sinapinic acid saturated in acetonitrile/H₂O (50:50). 0.5 ul of sample solution and 0.5 ul of matrix solution were mixed and placed on the laser target.

[0184] Dalbavancin binds to the protein as the monomer (1 HSA + 1 dalbavancin). At very high dalbavancin to protein ratios (1:2, 1:10), the presence of complexes containing 2 molecules of dalbavancin per protein molecule can be observed.

Example 8. Isothermal Titration Calorimetry of Binding of Dalbavancin to N,N'-diacetyl-Lys-D-Ala-D-Ala in the presence of Human Serum Albumin

[0185] The binding of dalbavancin to *N,N'*-diacetyl-Lys-D-Ala-D-Ala, a peptide analog of cell-wall targets of dalbavancin, was investigated by isothermal titration calorimetry (ITC) in the presence of HSA over a range of concentrations (up to 600 µM) at 25°C, with some additional measurements at 37°C. HSA increased the solubility of dalbavancin and reduced its binding affinity for the tri-peptide ligand. Results were compared with those for vancomycin. The observed effects plateaued at relatively low HSA concentrations, consistent with a non-competitive binding model

that allows binding of ligand to dalbavancin both free in solution and (more weakly) to the dalbavancin-HSA complex.

[0186] Preliminary experiments demonstrated that, in the absence of serum proteins, dalbavancin and vancomycin show similar binding profiles: both give exothermic binding to *N,N'*-diacetyl-Lys-D-Ala-D-Ala, but no evidence of binding to dipeptide (D-Ala-D-Ala) or to Lys-D-Ala-D-Lactate. For dalbavancin/tri-peptide interaction, the data were consistent with binding with $K_{\text{diss}} = 1\text{-}10\ \mu\text{M}$, depending on temperature, similar to vancomycin under the same conditions. In the presence of HSA, the solubility of dalbavancin is significantly increased and the binding affinity for tripeptide is reduced in a manner consistent with competitive or non-competitive binding by HSA for the antibiotic. The experiments described in this Example were designed in order to: (a) compare dalbavancin/tri-peptide measurements at different temperatures (25 and 37°C) and different HSA concentrations; (b) use these data to construct a binding model to compare with observed numbers.

[0187] Dalbavancin was supplied by Biosearch Italia. Other reagents were from Sigma: vancomycin hydrochloride (Sigma V-2002, fw 1485.7), *N,N'*-diacetyl-Lys-D-Ala-D-Ala (Sigma D-9904, fw 372.4), human albumin (HSA; Sigma A-3782; mw 69,366).

[0188] Antibiotics and peptides were dissolved in aqueous buffer (20mM Na phosphate, 150mM NaCl, pH 7.4) containing HSA, with gentle stirring immediately before each experiment. Peptide concentrations were determined by weight. Dalbavancin concentrations were determined either by weight or by UV absorbance using the molar extinction coefficients $\epsilon = 12430$ (dalbavancin, $A_{280}^{1\%} = 68.42$), $\epsilon_{280} = 6690$ (vancomycin). HSA concentrations were determined by UV absorbance (HSA, $\epsilon_{280} = 37,700$; $A_{280}^{1\%} = 5.44$). Spectra were recorded at room temperature in 1cm pathlength quartz cuvettes using Shimadzu UV-160A or UV-1601 spectrophotometers, with samples diluted quantitatively with buffer if necessary to give absorbance in the 0.1-1 A range.

[0189] Isothermal titration calorimetry was performed using a Microcal VP-ITC instrument at 25 °C and 37 °C using standard operating procedures. See, *e.g.*, Wiseman et al., *Anal. Biochem.* (1989) 179, 131-137; Cooper, *et al.*, *Philos. Trans. R. Soc. Lond. Ser. A-Math. Phys. Eng. Sci.* (1993) 345, 23-35; Cooper, A., Isothermal Titration Microcalorimetry in C. Jones, B. Mulloy and A. H. Thomas (Eds.), *Microscopy, Optical Spectroscopy, and Macroscopic Techniques*. Humana Press, Totowa, NJ, (1994) p 137-150; Cooper, A., *Microcalorimetry of Protein-protein Interactions* in J. E. Ladbury and B. Z. Chowdhry (Eds.); *Biocalorimetry: The Applications of Calorimetry in the Biological Sciences*. Wiley, (1998) p 103-111; and Cooper, A., *Curr. Opin. Chem. Biol.* (1999) 3, 557-563. Samples were degassed gently prior to loading to prevent bubble formation in the calorimeter cell. Each experiment typically comprised 25 x 10 µl injections of peptide solution (≈ 1 mM) into the calorimeter cell (volume ≈ 1.4 ml) containing antibiotic solution (≈ 20-100 µM). Control experiments involved injection of ligand into buffer under identical conditions to determine heats of peptide dilution, and these values were used for correction of raw binding data prior to analysis. Dalbavancin/tri-peptide binding experiments were repeated several times at each temperature. ITC binding data were analysed using standard Microcal ORIGIN™ software to determine the apparent number of binding sites (N), the binding affinity ($K_{\text{ass}} = 1/K_{\text{diss}}$) and enthalpy of binding (ΔH).

TABLE 17

Thermodynamic data for binding of tri-peptide binding to dalbavancin and vancomycin determined by ITC assuming a simple non-cooperative binding model: effects of temperature and HSA.

T	N	K_{ass}	K_{diss}	ΔH	ΔS	[HSA]
°C		M ⁻¹	µM	kcal/mol	eu	µM

<u>Dalba-</u> <u>vancin</u>	10	0.59	6.70E+0	1.49	-10.26	-9.60	0
			5				
	10	0.59	9.20E+0	1.09	-8.95	-4.30	0
			5				
	10	0.59	6.85E+0	1.46	-10.30	-9.60	0
			5				
	10	0.56	6.24E+0	1.60	-10.30	-9.90	0
			5				
	25	0.6	3.13E+0	3.19	-10.10	-8.90	0
			5				
	25	x	3.30E+0	3.03	-11.30	-12.60	0
			5				
	25	x	3.20E+0	3.13	-11.70	-14.00	0
			5				
	25	x	2.80E+0	3.57	-14.30	-23.00	0
			5				
	25	0.57	3.03E+0	3.30	-12.60	-17.00	0
			5				
	25	0.74	3.19E+0	3.13	-11.20	-12.50	0
			5				
	25	0.54	3.93E+0	2.54	-12.90	-17.60	0
			5				
<i>with HSA</i>	25	0.37	1.18E+0	8.47	-26.40	-65.50	13.6
			5				
<i>with HSA</i>	25	0.35	1.18E+0	8.47	-27.80	-70.10	13.6
			5				
<i>with HSA</i>	25	0.68	3.50E+0	28.57	-20.00	-46.20	34.2

			4				
<i>with HSA</i>	25	0.83	2.76E+0	36.23	-16.90	-36.50	80.7
			4				
<i>with HSA</i>	25	1.38	3.49E+0	28.65	-18.60	-41.70	104
			4				
<i>with HSA</i>	25	0.9	3.10E+0	32.26	-21.05	-50.00	288
			4				
<i>with HSA</i>	25	1.242	2.84E+0	35.21	-19.50	-44.90	430
			4				
<i>with HSA</i>	25	0.86	2.18E+0	45.87	-15.00	-30.40	601
			4				
	37	0.82	1.30E+0	7.69	-12.50	-16.80	0
			5				
	37	0.6	1.10E+0	9.09	-17.40	-33.20	0
			5				
<i>with HSA</i>	37	0.179	1.25E+0	80.00	-98.60	-299.00	482
			4				
<i>with HSA</i>	37	0.5	9568	104.52	-26.60	-67.60	516
<u>Vancomy-</u>	10	1.1	6.90E+0	1.45	-9.49	-6.80	0
<u>cin</u>			5				
	25	0.91	3.40E+0	2.94	-10.70	-10.60	0
			5				
	25	0.97	3.60E+0	2.78	-12.90	-17.80	0
			5				
<i>with HSA</i>	25	1.05	2.90E+0	3.45	-10.09	-8.80	513
			5				

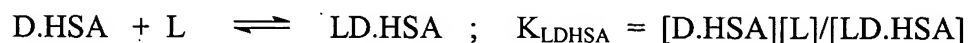
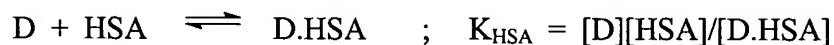
[0190] ITC experiments on the binding of tri-peptide to dalbavancin in the absence of HSA give consistent data for binding affinities, with average K_{diss} in the region of 1.4, 3.1, and 8.4 μM at 10, 25, and 37 °C respectively (**Table 17**). Binding is exothermic. Concentration calculations here indicate that N is closer to 0.5 under these conditions. This is consistent with the binding of one tri-peptide molecule per dalbavancin dimer under these conditions. These binding affinities and enthalpies of binding are comparable to those observed with vancomycin under the same conditions (**Table 17**, and D. McPhail, A. Cooper, *J. Chem. Soc.-Faraday Trans.* (1997) 93, 2283-2289.). Note also that vancomycin undergoes ligand-induced dimerization at higher concentrations.

[0191] Addition of HSA to the dalbavancin mixtures reduces the apparent binding affinity between tri-peptide and dalbavancin, though the binding is apparently more exothermic (**Table 17**). The HSA concentration dependence for this at 25 °C is shown in **Figure 11**. After an initial rise (weakening) in apparent K_{diss} up to 35 μM HSA, it remains relatively constant for higher HSA concentrations approaching physiological levels (600 μM). The plateau level at high concentrations of HSA ($K_{\text{diss}} \approx 35 \mu\text{M}$) corresponds to around 10-12x weaker binding affinity than in the absence of HSA. A similar reduction is seen at 37 °C.

[0192] The HSA effect was not due to interaction with the tri-peptide. Control ITC experiments for binding of vancomycin to tri-peptide in the presence of HSA gave results comparable to those seen in the absence of HSA (see **Table 17**). This indicates that neither the peptide nor the closely related antibiotic, vancomycin, interact with HSA in solution. It follows that any effect of HSA on dalbavancin/tri-peptide interaction must be due to interaction between HSA and dalbavancin.

[0193] Although not wishing to be bound by theory, the above data are consistent with a non-competitive binding model. This model assumes that the tri-peptide

ligand (L) can bind to dalbavancin (D) both in the free state and (possibly with different affinity) in the dalbavancin-HSA complex.



Apparent (observed) ligand binding dissociation constant (non-competitive):

$$\begin{aligned} K_{app,L} &= [total D][L]/[total DL complex] \\ &= ([D] + [D.HSA])[L]/([DL] + [LD.HSA]) \\ &= K_L \{1 + [HSA]/K_{HSA}\} / \{1 + [HSA].K_L/K_{HSA}.K_{LDHSA}\} \end{aligned}$$

[0194] This shows a hyperbolic dependence of $K_{app,L}$ on free HSA concentration that agrees well with the observed data (**Figure 11**). At high [HSA] this reaches an asymptotic (plateau) value

$$K_{app,L} = K_{LDHSA} \text{ (for large [HSA])}$$

[0195] This suggests that the binding affinity for tri-peptide is around 35 μ M when dalbavancin is bound to HSA, compared to 3 μ M for free dalbavancin (25 °C figures).

[0196] Further mechanisms may be suggested in terms of whether dalbavancin is acting as a monomer or a dimer in its interactions with peptides or proteins. Direct comparison of dalbavancin with vancomycin (which shows unambiguous 1:1 binding at these low concentrations) shows that binding is complete at lower molar ratios

(lower N) for dalbavancin (**Figure 12**). This is consistent with a 2:1 dalbavancin:peptide complex.

[0197] However, in the presence of HSA, the apparent N values increase (Table 17), and may be more consistent with 1:1 complexation. Although not wishing to be bound by theory, the model shown in **Figure 13**, showing the possible interaction of dalbavancin monomers and dimers with tri-peptide ligands and HSA, is consistent with these observations. **Figure 13A** depicts dalbavancin as in monomer-dimer equilibrium in solution (predominantly as dimer), but binding as monomer to two separate sites on HSA. This is consistent with the $N = 0.5$ values observed by ITC for binding of serum proteins to dalbavancin (Example 3). **Figure 13B** depicts ligand binding to the dalbavancin dimer in solution, and (more weakly) to the dalbavancin monomers attached to HSA. This is consistent with the non-competitive binding of dalbavancin to both tri-peptide and to HSA, with variable apparent stoichiometries.

[0198] In sum, this Example shows that HSA reduces the binding affinity of dalbavancin for tri-peptide ligand in a manner consistent with a non-competitive mechanism and that dalbavancin bound to HSA retains its ability to bind tri-peptide ligands, albeit with reduced affinity. These results are also consistent with a model in which dalbavancin is in monomer-multimer equilibrium, predominantly multimeric in solution, with strong affinity of multimers for peptide ligand. Dalbavancin monomers, both free and bound to serum albumin, may also bind peptides with a reduced affinity.

Example 9. A-40926 and Dalbavancin Preparations

Preparation of A-40926

[0199] A-40926, the natural glycopeptide produced by fermentation, was the starting material for producing Dalbavancin. It was produced by a *Nonomuria* sp ATCC 39727 as a mixture of A-40926 and its acetyl derivative (see U.S. Patent No.

4,935,238 and B. Golstain et al. *Antimicrobial Agent and Chemotherapy*, Dec. 1987, p.1961-1966). The acetyl derivative was first deacetylated to A-40926. After deacetylation, A-40926 was purified by column chromatography on polyamide as described below. The following description is representative of the current production method. The amounts reported here are about 1/4 of the amounts usually worked in an industrial preparation.

Deacylation of A-40926

[0200] 10 m³ of fermentation broth (23 °C) containing a total of about 1 g/L of A-40926 and its acetyl derivative were adjusted, under stirring, at pH 11.4 with NaOH 30 %. Stirring was continued for 6 hours, then the temperature was reduced to 15 °C and the broth was microfiltered (Koch Protosep IV Microfilter with a 0.12 m² ceramic membrane 0.1 μ). During microfiltration, water was continuously added to the retentate in order to obtain, at the end of the process, a permeate of 20-25 m³ and a retentate of 4.5-5 m³ (one half of the starting value).

[0201] The permeate, which contained A-40926, was analyzed by HPLC. When the deacetylation was completed, the pH of the permeate solution was adjusted at pH 7 with 30 % sulfuric acid (stored at 20 °C). In this example, 25 m³ of filtered broth containing 6.62 Kg of A-40926 (268 mg/L) was obtained. The deacetylation yield was 66.2 %. If the microfiltration process was carried out for longer time and higher extraction volume than those employed in this process, the yield can be increased up to 90 %.

Purification of A-40926 on polyamide column

[0202] After extraction, A-40926 contained in filtered broth was purified on a polyamide column, as described below. The amount reported in this description is about 1/10 of the amount usually worked in an industrial preparation and is representative of the current production method.

[0203] 500 L of polyamide resin SC6 (from Macherey Nagel) were suspended in demineralized water and loaded into a column. The resin was then conditioned at pH 6-6.5 by eluting the column with at least 2 BV (bed volume) of a buffer solution prepared by dissolving 4 Kg of sodium carbonate in 800 L of water and adjusting the pH of the resulted solution with acetic acid.

[0204] The A-40926 filtered broth (9000 L; assay 0.275 mg/L; A-40926 2475 g; pH 6 ± 0.2 ; temperature 10 ± 3 °C) was loaded into the column at about 5 g of activity per liter of resin (activity/resin ratio of 5-8 g/L was usually used). The column was washed with the following solutions: 3 BV (1500 L) of a solution at pH 6 prepared by dissolving 7.5 Kg of sodium carbonate in 1500 L of demineralized water and adjusting the pH with acetic acid; 4 BV (2000 L) of a solution at pH 8 prepared by dissolving 10 Kg of sodium carbonate in 2000 L of demineralized water and adjusting the pH with acetic acid; 1.5 BV (750 L) of a solution at pH 9 prepared by dissolving 4 Kg of sodium carbonate in 750 L of demineralized water and adjusting the pH with acetic acid.

[0205] A-40926 was recovered from the column by eluting with 4 BV (2000 L) of a buffer solution at pH 10 prepared by dissolving 10 Kg of sodium carbonate in 2000 L of demineralized water and adjusting the pH with acetic acid. The fractions containing purified A-40926 (concentration of A-40926 greater than 0.5 g/L and HPLC area % of the main component (Bo+B₁) greater than 80 %) were collected, neutralized with 1N HCl, and analyzed by HPLC. About 2000 L of final clear solution were obtained.

[0206] The resin used for the purification was regenerated with 1.5 BV of 1:1 mixture of isopropanol / 5 % NaOH followed by a washing with 5 BV of demineralized water.

A-40926 concentration

[0207] The solution coming from the column was subject to several rounds of dilution/concentration steps to eliminate most of the inorganic salts in the solution. The solution was concentrated to 80 L by nanofiltration using a membrane with a cut-off of 250 D, diluted with 80 L of demineralized water, and re-concentrated at the starting volume (80 L) by nanofiltration. This operation was repeated at least 5 times. The pH of the final solution (80 L, pH 7.5) was adjusted at pH 6.3 with 23 % HCl. The solution was then diluted with 80 L of acetone, and its pH was adjusted again at pH 2.6 with 23 % HCl.

Decoloring

[0208] 680 g of charcoal PA 200 C (~0.3g/g A-40926) was added under stirring to the solution obtained in the above step (160 L). Stirring was continued for at least 30 minutes at room temperature, then about 0.5-0.6 Kg of filter aid (DIF-BO) was added. The mixture was filtered through a filter cartridge. The clear solution obtained was concentrated under *vacuo* (45 °C) in order to reduce the acetone below 10 %. The final volume was about 100 L. The pH was then adjusted at 6.7 with aq. NaOH, and the concentration step was continued using the usual nanofilter until the A-40926 concentration was around 100 g/L. 20 L concentrated solution was obtained (A-40926 1884 g, 94.2 g/L).

Precipitation and drying

[0209] The previous solution was diluted under stirring with 20 L of acetone, and its pH was adjusted at 5.1 with 10 % HCl. To this solution additional 5 volumes of acetone (100 L) were added to complete the A-40926 precipitation. If water content was not <15% at this point, additional acetone was added. After 2 hours the suspension was centrifuged, and the solid was washed with 3 x 10 L of fresh acetone. The mother liquors were analyzed and discharged after having ascertained the absence of product.

[0210] Solid A-40926 was dried under reduced pressure at 30-35 °C in a static drier until the residual acetone was below 2 % and the water was less than 10 %. The product was then sieved through a 50 mesh sieve obtaining 2.08 Kg of purified A-40926 (HPLC assay 81.4 %; water 6.2 %; sulphated ashes 4.8 %). The yield, starting from the activity loaded on the column, was 68.4 %.

Synthesis of Dalbavancin

[0211] Dalbavancin (BI-397) was prepared from the natural glycopeptide A-40926 through a three-step synthesis as described in Malabarba and Donadio (1999), *Drugs of the Future*, 24(8):839-846. Specifically, A-40926 was first subject to an esterification step to make MA, which was then subject to an amidation step to make MA-A-1. A final hydrolysis step then converted MA-A-1 into dalbavancin.

Esterification step (Step 1)

[0212] The following description is representative of the current method in use.

Preparation of H₂SO₄ 96%/MeOH (Solution A)

[0213] In a 15 L round bottomed flask equipped with a mechanical stirrer and a thermometer, 2.28 L of H₂SO₄ 96% (~300 mL of H₂SO₄ 96% per Kg of A-40926 powder) was added drop wise to 7.9 L of MeOH. An external ice bath was used to maintain the temperature between 0 and 5 °C.

Reaction procedure

[0214] Starting material A-40926 (7.6 kg; batch 019, assay 85.09 %; activity 6.46 kg; 3.73 mol) was suspended in a 140 L glass-lined reactor in MeOH (46 L), and the resulting suspension was cooled at 0 °C ± 2 °C. At this temperature the suspension was treated with the previously prepared Solution A (H₂SO₄/MeOH). The resulting solution was stirred at 0 °C for 22-26 hours while the reaction (a reaction aliquot diluted 100 times with 1:1 acetonitrile/water mixture) was monitored by

HPLC analysis every two hours. The esterification was considered complete when the residual A-40926 was less than 5 % and diester was not more than 10 % as HPLC area %.

Ester (MA) isolation

[0215] When the reaction was complete, the mixture was cooled at -5 °C (+/- 2°C) and diluted with a same volume of cold water (54 L) maintaining the temperature below 5 °C. The product (MA) was precipitated by adjusting the pH of the solution at 5.5 (+/- 0.2) by slowly adding 10.2 L of triethylamine (TEA). Stirring was continued for an additional hour at 0-2 °C, then the solid obtained was centrifuged, washed with water (10 L per Kg of A-40926) and finally with MeOH (3 L of MeOH per Kg of starting A-40926) previously cooled at 10-15°C. Washing with water was done primarily to remove sulphates from MA.

[0216] Mother liquors and washings were separately analyzed and discharged if contained less than 1-2 % of activity. The product was dried *in vacuo* (50 mmHg) at 35-40 °C (external temperature) until the residual water was less than 10 %. 7.6 Kg of MA (5.63 kg activity, 3.23 mol) was obtained as a brownish powder.

[0217] The analysis showed the following values of HPLC area %: MA 89.8, A-40926 3.2, Diester derivative 5.9. The HPLC assay was 74.2 %, activity 5.637 Kg; 3.23 mol; yield = 86.5 %. This material was used in the following step without any further purification.

Amidation step (Step 2)

[0218] The following description is representative of the current production method.

Preparation of the DMSO / HCl mixture (Solution B)

[0219] DMSO (1.6 L) was placed into a 10 L round bottomed flask, equipped with a mechanical stirrer and a thermometer, and cooled with an ice bath below 10

°C. HCl 37% (1 L) was then slowly added under stirring maintaining the temperature of the mixture below 25 °C.

Amidation procedure (production of MA-A-1)

[0220] Starting material MA 5.95 kg (assay 76.3 %, KF 8.9 %; 2.68 mol) was slowly dissolved under stirring in 19.2 L of 1:1 DMSO / MeOH mixture (~1.6 L DMSO and 1.6 L MeOH per Kg of MA powder) at room temperature. After 1 hour of stirring 709 mL of 3-(dimethylamino)-propylamine (DMEPA, MW 102.1; density = 0.812 g/mL; 5.63 mol; 1.96 mols per mol of starting MA) and 325 g of 1-hydroxybenzotriazole hydrate (HOBTH₂O; MW 153.1; 2.04 mol; 0.71 mol per mol of starting MA) were added to the reaction mixture. Stirring was continued until a complete solution was obtained, then the mixture was adjusted at pH 3-3.1 (measured after diluting an aliquot of the reaction 10 times with water) by slowly adding about 2.0 L of Solution B (DMSO/HCl).

[0221] A dicyclohexylcarbodiimide (DCC) solution, prepared by dissolving 1.03 Kg of DCC (4.99 mol; MW 206.3; 1.74 mol per mol of MA) in 4.1 L of 1:1 DMSO/MeOH mixture, was added to the stirred reaction mixture in 10 minutes. Stirring was continued for 5 hours, then additional 51.5 g of solid DCC (0.25 mol) was added to the reaction mixture in order to lower the residual MA under 5%, maintaining the level of isoureas lower than 4-5 %. Isoureas are a group of by-products produced by further reaction of Dalbavancin with the excess of DCC.

[0222] Typically after 2 additional hours (7 hours total) the reaction was completed. At the end the mixture was diluted with water (60 L) to lower the DMSO concentration to 15 % (v/v) and adjusted at pH 2.3 with HCl 1N (0.85 L) to destroy any residual DCC.

Hydrolysis of MA-A-1 to Dalbavancin (Step 3)

[0223] After 30 minutes the mixture was adjusted at pH 12.0-12.1 with 15 % NaOH (8 L). Stirring was continued for 4 hours maintaining the mixture at this pH

with small additions of NaOH 15%. After this time the residual MA-A-1 was less than 0.2 % as HPLC area %.

[0224] The mixture was then acidified at pH 3.0 with 1N HCl (19 L), and the suspension was filtered to remove the dicyclohexylurea formed. The solid cake was washed on the filter with demineralized water (2x20 L). Washings and filtrate were gathered together, obtaining a clear solution which was analyzed by HPLC. 152.8 L of solution containing 21.74 g/L of Dalbavancin (total activity 3322 g; 1.828 mol, yield = 68.2 %) was obtained.

Purification of dalbavancin

[0225] The following description is representative of the current production method.

[0226] The 152.8 L of solution obtained from the hydrolysis step and containing 3322 g of Dalbavancin activity was split into two parts and each one was purified separately on the same chromatographic column containing 400 L of polyamide. In these two purification runs the activity/ resin ratio were 4.3 and 4.0 g/L respectively.

Polyamide column preparation

[0227] The glass-lined column (internal diameter = 40 cm, h = 320 cm) containing 400 L of polyamide resin was cleaned according to the resin regeneration procedures (see below) and conditioned with 2 BV (800 L) of demineralized water acidified with 4 L of AcOH (pH = 3.2).

Purification of the first portion

[0228] The first portion of 76.4 L of starting solution was diluted with H₂O (56 L) in order to lower the DMSO content under 5% (v/v), and acidified to pH 2.78 with 1 N HCl (3.4 L). This solution was then loaded onto the column at a flow rate of 150 L/h. After loading, the resin was washed with the following solutions: 4 BV (1600 L) of H₂O acidified with AcOH (8 L), pH = 3.2; 5 BV (2000 L) of AcONa 0.1 M, pH =

8.2; 1 BV (400 L) of H₂O acidified with AcOH (1 L), pH = 3.2. Dalbavancin was eluted with 4 BV (2400 L) of H₂O/MeOH (8:2) acidified with AcOH (6 L), pH = 3.4.

[0229] During the elution step, 22 fractions of 50-60 L each were collected and analyzed by HPLC. Fractions from 9 to 25 (concentration of Dalbavancin higher than 0.5 g/L and HPLC area % of (B₀+B₁) ≥80%) were pooled together, obtaining 969 L of solution with 1.56 Kg of Dalbavancin (yield = 93.9 %). This solution was then concentrated by nanofiltration, obtaining 125.7 L of solution with 1.38 Kg of Dalbavancin. 850 L of permeate, containing 145 g of impure Dalbavancin (8.7 %), were neutralized and discharged.

Resin regeneration

[0230] Before re-using the resin was washed with the following solutions: 2.5 BV (1000 L) of 1:1 MeOH /water acidified with acetic acid (2.5 mL/L); 2.5 BV (1000 L) of 1:1 0.5 % NaOH / isopropanol; 10 BV (4000 L) of demineralized water. The resin was then re-equilibrated with BV (800 L) of water acidified with acetic acid (2.5 mL/L).

Purification of the second portion

[0231] The second portion of the starting solution coming from the hydrolysis step (76.5 L) was diluted with H₂O (56 L) to lower the DMSO content under 5% (v/v) and acidified to pH 2.87 with 3.0 L of 1 N HCl. The portion was then purified as previously described in the purification of the first portion. The pooled fractions (vol. = 972 L, Dalbavancin 1.54 Kg, yield = 92.7 %) were concentrated by nanofiltration, obtaining 133 L of solution with 1.46 Kg of activity. 850 L of permeate, containing 73 g of Dalbavancin (4.3 %), was discharged.

[0232] The concentrated solutions coming from the two purification steps were re-analyzed and pooled together giving 258 L of solution containing 2840 g of purified Dalbavancin. The purification yield was 86 %. The total yield, starting from MA, was 58.3 %.

Final polyamide regeneration

[0233] After the second purification run polyamide was regenerated with: 2.5 BV of 1:1 MeOH-water, acidified with AcOH (2.5 L) at pH =3.4; 2.5 BV of 1:1 0.5 % NaOH-isopropanol; 10 BV of demineralized water.

Decoloration and precipitation of Dalbavancin

[0234] 1.5 mol of 1N HCl per mol of Dalbavancin and 0.3 g of charcoal CG1 (0.85 Kg, from NORIT) per gram of Dalbavancin were added to the 258 L solution obtained above. The mixture was stirred at room temperature for at least 45 min. The pH was 3.1 . The suspension was then filtered on a SUPRA DISC cartridge mod. SDP-EK1 from SEITZ-SCHENK, and the cake was washed with 50 L of H₂O/MeOH 8:2. The filtrate was analyzed and concentrated again by nanofiltration, using a MPS 44 membrane with a cut off of 250 D. 21.3 L of concentrated solution containing 119 g/L of Dalbavancin (pH 4.1; MeOH 1.9 %,GC) was obtained. 909 mL of 1 N HCl was finally added to adjust the pH at 2.63, which corresponds to the salification ratio of 1.65 mol_{HCl}/mol_{Dalbavancin}.

[0235] The solution (22.2 L) was poured out, under stirring, in 200 L of acetone. The solid obtained after decanting was centrifuged and washed with 14 L of fresh acetone. The product was then dried under reduced pressure (50 mmHg) at 35°C, maintaining the internal temperature under 30 °C for 17 hours. During the drying process, 1 L of pyrogen-free water (<250 EU/mL), divided in two portions of 0.5 L each, was sprayed on the solid after three and five hours in order to remove the residual acetone that otherwise is difficult to eliminate. The product was then sieved (50 mesh), obtaining 2592 g of Dalbavancin (HPLC Assay 82.4 %; water (KF) 14 %; Cl⁻ 3.0 %).

Example 10. Alternative Methods for A-40926 and Dalbavancin Preparation

[0236] The methods described below are alternative methods that can be used in the A-40926 and Dalbavancin preparation process.

A-40926 Preparation on XAD-7 HP

Deacetylation and mycelium microfiltration

[0237] 150 L of fermentation broth containing A-40926 (pH 7) were stirred in a suitable reactor at room temperature (24 °C), and adjusted at pH 11.5 with a 2.5 N NaOH solution (2.5L). After 4 hours of stirring, the broth was adjusted at pH 10.6 with 15 % HCl, and microfiltered through a 0.2 micron membrane. 439 L of clear permeate was collected and then concentrated by nanofiltration using a MPS 44 membrane with a 250 D cut off. The A-40926 concentrated solution obtained (58.6 L; 3.89 g/L) was adjusted at pH 6.4 and stored at 4°C until used.

Column preparation and purification.

[0238] XAD-7 HP resin (8L) was suspended in a 1:1 water/methanol solution, filtered, and loaded into a proper glass-column (internal diameter 12 cm) with a peristaltic pump.

[0239] The resin was then washed with water and equilibrated with 6 BV of a sodium carbonate aqueous solution buffered at pH 6 prepared by dissolving 5 g of sodium carbonate per liter of water and adjusting the pH with acetic acid.

[0240] A portion of concentrated broth containing 194 g of A-40926 was loaded into the XAD-7 HP column. The resin was then washed with the following two buffered solutions, at a flow rate of ½ BV /hour, in order to eliminate part of the hydrophilic and the colored substances present: 3 BV (24 L) of aq. 0.5 % Acetic Acid

solution adjusted at pH 5 with 30 % sodium hydroxide; 5 BV (40 L) of a 8:2 mixture water/acetone with 5 mL of acetic acid / L of water.

[0241] A-40926 was finally eluted with 8 BV (64 L) of a 1:1 water/acetone mixture acidified with 5 mL of acetic acid / L of water. 16 fractions of 4 L each were collected. The rich fractions (from 5 to 15) in which A-40926 concentration was greater than 0.5 g/L were gathered together obtaining a solution containing 163.4 g of A-40926 (43 L, 3.8 g/L). The column yield was 81.3 %. The other fractions (200 L) containing 0.23 g/L (45.3g; 22.2 %) of less pure A-40926 were discharged.

[0242] After the elution the resin was regenerated with 6 BV (55 L) of NaOH 0.5% / isopropanol (1:1) mixture, and finally washed to neutral with 10 BV of water.

Charcoal treatment.

[0243] The collected fractions were adjusted at pH 2.5 with HCl 37% (70 mL) and then decolorized with 50 g charcoal type PA 200 (0.3 g/g of A-40926). The suspension obtained was stirred for 2 hours at room temperature and then filtered through a KS 50 filter (d=25 cm, time = 2.5 hours), obtaining 45.6 L of a slightly yellow A-40926 solution (3.5 g/L; yield=96.4 %).

Concentration.

[0244] The decolorized solution was adjusted at pH 7 with NaOH 30% (230 mL) and concentrated by nanofiltration and ultrafiltration. The use of these techniques was important for the elimination of the hydrophilic substances that were detected on the HPLC chromatograms at Rt = 2-4 minutes. When the retentate was concentrated to 1/10 of the starting volume (4 L), the same volume of water was added and the solution obtained was concentrated again. This concentration/dilution step was repeated three times in order to reduce the residual acetone to 0.25 %. The final solution (2.2 L, 146.3 g of A-40926, 66.5 g/L, yield=91.5%) was analyzed by HPLC. The purification yield was 75.4 %.

A-40926 crystallization.

[0245] A 300 mL portion of the A-40926 solution (19.9 g of A-40926) was further concentrated to 100 mL by using a laboratory scale ultrafilter and then heated at 60-65 °C. The pH of this solution was adjusted at 7 (30% NaOH), and 1.2 mL of 5:1 acetone/isopropanol mixture per mL of concentrated solution was added drop wise at this temperature. The resulted mixture was left to cool at 20 °C. After 1.5 hours, the solid obtained was filtered, washed on the filter with acetone, and dried at 40 °C for 15 hours. 20.6 g of product (HPLC assay 82.0 %; A-40926 16.9 g) was obtained. The precipitation yield was 84.9 %. The overall yield, starting from the filtered broth, was about 64 %.

A-40926 Preparation on CG -71

Column preparation

[0246] CG-71 resin (350 mL) was poured into a glass-column (internal diameter = 4 cm) and washed with water. The resin was equilibrated with 3 BV of a sodium carbonate solution, prepared by dissolving 5 g of sodium carbonate in water at pH 6 with acetic acid. 250 mL fermentation broth (pH 7) containing 14.7g of A-40926 was loaded into the column (42 g/L resin). The resin was washed with the following three solutions: 1050 mL (3 BV) of aqueous solution of sodium carbonate (5 g/L) adjusted at pH 6 with acetic acid; 1750 mL (5 BV) of aqueous solution of sodium carbonate (5 g/L) adjusted at pH 8 with acetic acid; 3150 mL (9 BV) of aqueous solution of sodium carbonate (5 g/L) adjusted at pH 9 with acetic acid.

[0247] The activity was then eluted with 10 BV of demineralized water. 20 fractions of 500 mL each were collected. Fractions 12 to 15 were pooled together, obtaining 2.2 L purified solution containing 11.7 g of A-40926 (yield = 79.6 %). This solution was then concentrated by ultrafiltration and the concentrate solution

was further diluted with demineralized water and ultrafiltered again. The solution obtained was further concentrated under reduced pressure to 50 mL.

A-40926 crystallization.

[0248] The concentrated solution was heated at 60 °C and treated under stirring with a 5:1 acetone/IPA mixture (60 mL). The mixture was then slowly cooled at room temperature. The solid obtained was filtered, washed with acetone on the filter, and dried under vacuum at 35 °C for 80 hours. 8.9 g of purified A-40926 (HPLC assay 84.2 %) was obtained. The overall yield was 51 %.

Alternative amidation step in dalbavancin synthesis using N-Methyl-2-pyrrolidine (NMP) as a solvent

[0249] MA mixture was added portion wise under stirring to a 1:1 NMP/MeOH mixture (64 mL). Stirring at 20-25 °C was continued until complete solution, then DMEPA (2.42 mL; 1.96 mol/eq_{MA}) and HOBT (1.06 g; 0.71 mol/eq_{MA}) were added. The pH of the reaction mixture (checked on a sample diluted 1:10 with water) was adjusted to 3.0 with 9.37 mL of 15% HCl in NMP (previously prepared from 34.0 mL HCl 37% dissolved in 57.7 mL NMP). Then a solution of DCC (3.17g; 1.57 mol/eq_{MA}) in NMP/MeOH 1:1 (12.7 mL) was added under stirring. The reaction was monitored by HPLC. The reaction was complete after about 6 hours (MA-A-1 88.9%, MA 7.3%, ISO 3.7%). This experiment suggests that NMP can be a convenient alternative to DMSO for the amidation reaction. The whole process was not influenced by this solvent change and the final Dalbavancin obtained was chemically equivalent to other batches.

Alternative method of Dalbavancin preparation: One-pot procedure

[0250] 10 g of A-40926 complex (HPLC titer 80.66%, 4.6 mmole) was suspended in 24 mL of MeOH under stirring at room temperature in a 100 mL glass

reactor. The mixture was cooled at 0°C, and a solution of 4 g of HCl (g) in 16.4 mL of MeOH was added to complete the product solubilization. The temperature was then left to rise to 20°C while stirring was continued for additional 24 hours.

[0251] After this time, 40 mL of DMSO and 0.4 g of HOBT were added to the reaction mixture.

[0252] 1,1-dimethylamine propylamine was then added, adjusting the pH of the resulting reaction mixture between 3-3.1 (measured after diluting a sample 9:1 with water). 1.8 g of solid DCC was then added and stirring was continued for additional 15 hours. After this time the reaction mixture was transferred in a 1 L glass reactor and diluted with 80 mL of water. The pH was then brought to 12 by adding 240 mL of 15% NaOH. Stirring was continued for additional 60 minutes, and the mixture was acidified at pH 2.8 with 260 mL of 15% aq. HCl. About 800 mL of final clear solution containing 6.4 g of Dalbavancin was obtained (yield = 76%).

[0253] HPLC analyses showed that the profile of the product obtained is comparable with that obtained with the other manufacturing processes.

* * * * *

[0254] Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced without departing from the spirit and scope of the invention. Therefore, the description should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

[0255] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes and to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference.